1997 PROGRESS REPORT ON FOOD SAFETY RESEARCH CONDUCTED BY ARS

Agricultural Research Service

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EXECUTIVE SUMMARY

The safety of the Nation's food supply is one of the most important issues to American consumers. Consumers are demanding a safety assurance system for raising, harvesting, processing, transportation, handling and storage of food emphasizing prevention of foodborne pathogens. This need to assure food safety was recognized by the Federal government in the Food Safety Initiative (FSI) of 1997 which calls for expanded food safety research and risk assessment efforts. The goal of the FSI is to prevent food contamination from production to consumption **(Farm to Table Continuum)**; the FSI emphasizes the following areas of risk assessment and food safety research: 1) Develop and validate **pathogen exposure assessment models**; 2) **Development of rapid, cost-effective tests for the presence in foods of pathogens** such as *Salmonella*, *Cryptosporidium*, *E. Coli* 0157:H7 and others; 3) **Enhance understanding of how pathogens become resistant** to food-preservation techniques and antibiotics and 4) **Develop technologies for prevention and control of pathogens,** such as new methods of decontamination of meat, poultry, seafood, fresh produce and eggs. Agricultural Research Service (ARS) has the appropriate physical facilities, state of the art laboratory equipment and the scientific expertise and experience to make significant contributions to all of the above important aspects of food safety research and risk assessment.

This report summarizes ARS research progress on Food Safety of animal products in 1997. It does not yet include the ARS studies to control pathogens on fruits and vegetables. The research is categorized into 4 general areas: **I. Control of Foodborne Pathogens in Live Animals; II. Pathogen Control During Slaughter and Processing (Inspection Technology); III. Post-slaughter Pathogen Modeling and Control; IV. Residue Detection and Chemical Analysis.**

I. Control of Foodborne Pathogens in Live Animal: Food safety of animal products begins with management practices, including herd and flock health programs to prevent disease and control infection in the live animal. ARS research to control human pathogens in live animals includes vaccine development, competitive exclusion cultures, and breeding and selection of resistant animals. This research benefits greatly from the long ARS experience with zoonotic pathogens in large animals. ARS has developed two competitive exclusion cultures (CEC) to control *Salmonella* on commercial broiler farms and the technologies have been transferred to CRADA partners and are awaiting Food and Drug Administration (FDA) approval. The use of similar products to control *Salmonella* in turkeys is being investigated. Another CEC has been developed to control *Salmonella* in swine and an investigational new animal drug application has been submitted to the FDA.

Certain phage types (PT) of *Salmonella enteritidis* (SE) are routinely isolated from contaminated eggs, hen-house environments, rodents and from infected people. However, PT4, which is a particularly virulent SE, and has been historically associated with incidence of illness in people in other countries, has now been isolated in the U.S. ARS identified the outer

membrane characteristics unique to PT4 SE. This assay can be used by FSIS and diagnostic labs to group all SE isolates into PT4 and non-PT4 lineages.

As part of a National Antimicrobial Susceptibility Monitoring System *Salmonella* isolates obtained from cattle, swine, chickens, turkeys, swine feed, ground product, exotics, dogs and cats were tested for susceptibility to 16 antimicrobials. All isolates were susceptible to amikacin, cepotaxime, and ciprofloxacin. Approximately 34% and 28% of the isolates were resistant to tetracycline and sulfamethoxazole, respectively, while 13% of the isolates were resistant to both ampicillin and ticarcillin. Less than 9% resistance was observed for all other antimicrobics.

A T-cell line has been developed which is capable of producing large quantities of *Salmonella enteritidis* (SE)-immune lymphokine (VILK). Two patents have been allowed for a T-cell line to produce VILK and for *in ovo* administration of VILK for prevention of disease. A CRADA has been formed for the development and use of VILK.

A continuous-culture model of rumen micro flora has been developed to predict growth and survivability of *E. coli* O157:H7 in fasted and well fed cattle. *E. coli* O157:H7 was demonstrated to colonize the intestines of cattle and to be shed in the feces. Newborn calves may be very susceptible to the infection and develop severe diarrhea while weaned calves are more resistant to the disease. Virulence factors of *E. coli* O157:H7 and other Shiga toxin-producing *E. coli* have been identified and both intimin and Shiga toxin were shown to have a role in colonization in weaned calves but only intimin was required for pathogenesis in newborn calves. Vaccinating cattle for *E. coli* O157:H7 may be possible, however, this may be difficult because shiga toxins were shown to be immunosuppressive.

Methods are being developed for subtyping of *Campylobacter* Strains. A new oral vaccine to prevent *Campylobacter* colonization of chickens is being tested.

Farm audits are being conducted in cooperation with APHIS to determine trichinae infection rates and to certify farms free of infection. A national prevalence assessment of trichinellosis in hogs is being conducted which will be used in decisions of control and risk assessment.

II. Pathogen control during slaughter and processing (Inspection Technology): Slaughter and processing is a key link in the food safety chain. Improved understanding of foodborne pathogen transmission, control steps in prevention, sanitation, and processing technology are necessary elements to prevent contamination, cross contamination and wide spread foodborne illness. ARS research in this area includes the isolation of over 15 different serovars of *Salmonella* from hog pen floors and hog hauling trailers. Thus, washing and sanitizing hog hauling trailers and holding pens has the potential to reduce contamination at the slaughter plant.

Feed withdrawal in broilers prior to slaughter increased infection rates of their crops with *Salmonella* and *Campylobacter*. Since crop removal and subsequent rupture of contaminated crops is a major source of carcass contamination, feed withdrawal prior to broiler processing represents a preharvest critical control point. Feed withdrawal used to induce molt to stimulate egg laying in aging flocks may decrease resistance to SE and increase risk to the nearly 180 million hens in systems using this practice. Methods, such as lactose in drinking water to restore SE resistance in molting hens, are being investigated.

Methods of fecal removal, defeathering, carcass scalding techniques and carcass dipping in acid/base dips are all being investigated to help reduce slaughter plant contamination. Optimum treatments will be determined and new recommendations will offer aid to the development of HACCP plans for commercial processors. Methods are being studied to understand formation and composition of biofilms on processing plant surfaces and to prevent their formation and allow more efficacious cleaning and sanitizing. Chemical and physical techniques of surface modeling to identify pathogen attachment to poultry are being investigated, and new compounds and methods are being identified that will interfere with pathogen adhesion on food surfaces and prevent formation of biofilms. Rubber picker finger material was found to be the surface least conducive to bacterial attachment.

The bacteriocin, Nisin was incorporated into a polyethylene based plastic film at 1 and 0.5 mg of nisin per ml of formulation. These plastics used for vacuum packaging of refrigerated meat subprimals reduced microbial counts throughout the 20 day test storage period.

An on-line inspection system using visible/near infrared (VIS/NIR) spectrophotometry to classify wholesome and unwholesome poultry carcasses is now pilot scale and can handle up to 100 birds per minute. An industrial prototype is ready for in-plant testing.

III. Post Slaughter Pathogen Modeling and Control: ARS research is developing an understanding of the thermotolerance of *E. coli* O157:H7, *Clostridium perfringens* and *Listeria monocytogenes* under different conditions of pH, acidity and growth temperature. Research demonstrated that parameters for heat treatment in lean turkey can be used to validate the safety of a process for controlling *E. coli* O157:H7 in lean pork and lamb. Research on the thermotolerance of *E.coli* O157:H7 defined the time and temperature needed to inactivate this pathogen and determined that heat shocking allowed the organism to survive longer than nonheat-shocked cells. Similarly, the influence of pH, acidulant and growth temperature history of *Listeria monocytogenes* on its heat resistance was determined. This work is essential in defining the effects of environmental conditions during growth for modeling studies and in designing thermal processes that ensure safety of ready-to-eat foods.

Using isolates of *Salmonella* from broiler operations it was determined that automated ribotyping discriminates between isolates better than serotyping. However, because of its limited ability to identify *Salmonella,* ribotyping cannot replace serotyping. Therefore, it is recommended that serotyping and ribotyping should be used together in epidemiologic investigations of *Salmonella* contamination.

Acceptance of irradiation will be enhanced by the availability of methods indicative of irradiation treatment. Methods utilizing either supercritical fluid extraction (SFE) and/or micro column chromatography show promise in identifying radiolytic products of riboflavin and hydrocarbons indicative of irradiated meat and poultry products.

Surface pasteurization techniques have been developed to reduce microbial contamination on the surface of solid foods without loss of quality. A prototype design to steam fresh whole broiler carcasses briefly, so that surface organisms are killed but with no appreciable cooking of the meat, was built, tested and patented. This treatment is sufficiently rapid that a single machine can serve 4000 birds per hour, which is the current rate of a modern slaughter line. Low temperature

electrical pasteurization for temperature sensitive liquid foods like liquid eggs offers exciting opportunities for pathogen control while maintaining product quality. A redesign of the electrical egg pasteurizer together with realization of pilot plant facilities for handling pathogens will speed significant progress in egg pasteurization.

Microbial models for risk assessment of foodborne pathogens including multiple strains of *E. coli* O157:H7, *Listeria moncytogenes* and *Shigella flexneri* have been developed. The *Salmonella* risk assessment in eggs will be the first foodborne pathogen risk assessment specifically created for regulatory purposes.

IV. Residue detection and chemical analysis: Food safety research also includes the detection of residues of drugs, environmental toxins, natural toxins and chemical analysis of nutrient quality. ARS research on methodologies for detection of drugs and their residues include supercritical fluid extraction techniques (SFE) with broad applications for extraction and detection of target chemicals while reducing solvent use and disposal problems. SFE was used to detect sulfonamides in chicken, beef, pork and eggs. The same technique is useful in identifying amphenicol drug residues in tissue and eggs, isolation of pesticide residues, dioxins and triazine herbicides in eggs. SFE has been used in nutrient analysis to identify contaminant marker compounds in fire-exposed meat, aged vegetable oil and flavor precursors.

Residues of chlorinated dioxins and furans in beef, chicken, and animal feeds and forages were detected and quantified, and most significantly, high dioxin/furan levels in beef were associated with animal exposure to pentachlorophenol treated wood. ARS research confirmed the high levels of dioxins in chickens that resulted from ball clay that was added to soybean meal as an anti-caking agent. Identification of these dioxin sources provides producers with the knowledge needed to produce dioxin-free food products. Studies of the disposition of the β-agonist clenbuterol have identified metabolites of clenbuterol and determined that they can be converted back to the parent drug in the GI tract, potentially posing a possible risk in humans consuming meat from exposed animals.

Development of monoclonal antibody based immunoassays are being evaluated for a number of compounds including: ceftiofur, fluoroquinolones, halofuginone, hygromycin B, 4,4'-dinitrocarbanilide, and various sulfa drugs. A monoclonal antibody capable of binding Hygromycin B was formatted into a rapid ELISA assay, and a patent was issued and the antibodies licensed to a private kit manufacturing company. CRADAs with industrial partners interested in immunoassay for the other drugs are being pursued.

Conclusion: ARS has an organized and productive research program on food safety of animal products. This research addresses control and prevention of pathogens and residues at every aspect of the **farm to table continuum;** from prevention in the live animal, to techniques of eliminating pathogens at the processing level, to storage, quality and preparation of food products. The ARS research program in food safety is of vital importance to FSIS and supports their efforts to continue to provide the consumer with the safest food supply in the world.

1997 PROGRESS REPORT ON FOOD SAFETY RESEARCH CONDUCTED BY ARS

(Pathogen Reduction and Residues in Food Products of Animal Origin)

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Part I. CONTROL OF FOODBORNE PATHOGENS IN LIVE ANIMALS

CYTOKINE-MEDIATED MODULATION OF THE INNATE IMMUNE RESPONSE TO PREVENT SALMONELLOSIS IN POULTRY

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OBJECTIVE A: Develop a non-traditional immunological method to prevent and/or control salmonellosis in poultry, optimize an effective delivery system(s) for our *Salmonella enteritidis*-immune lymphokine (VILK) and identify and purify the effector cytokine(s) and clone the gene for mass production.

PROGRESS A: During the past year, we have developed a T-cell line which is capable of producing large quantities of our *Salmonella enteritidis*-immune lymphokine (VILK). Administration of VILK into day-old poultry (chickens and turkeys) induces a protective innate response by these birds against infectivity by various serovars of paratyphoid and typhoid *Salmonella*. The protection persists for a minimum of 5-6 days with no evidence of any detrimental host pathology induced by the inflammatory reactions. In addition to inducing direct protection against salmonellosis, we have also found that administration of the VILK effectively controls the horizontal transmission of *Salmonella* from contaminated chickens and turkeys to contact birds.

This year tests were performed to evaluate whether VILK could be administered to day-old poultry by routes routinely used by the industry for vaccines and still be able to confer protection to the birds against localized enteric *Salmonella* infections. The results indicated that the delivery of VILK either orally, subcutaneously, or by aerosol confers significant protection against salmonellae infections that persists for up to six days.

Using antibodies against the human cytokine Granulocyte-Colony Stimulating Factor (G-CSF), we have tentatively identified G-CSF as a major effector molecule in VILK. We have also developed monoclonal antibodies against VILK which can neutralize various functional activities of VILK in neonatal poultry. These antibodies will be used for a) purification of VILK effector molecules using affinity chromatography and b) screening a cDNA library which has been generated from the mRNA isolated from the T-cell line which produced VILK.

IMPACT/TECH TRANSFER A: A CRADA has been formed with Eli Lilly and Company for the development and use of VILK for the control of poultry diseases. A patent for the *in ovo* administration of SE immune lymphokine for the prevention of infectious diseases of poultry was allowed this year. A second patent was allowed for the production of the T-cell line to produce VILK.

These experiments suggest that VILK could be readily administered to poultry without new specialized equipment. We are working with Eli Lilly to analyze the most cost effective route to administer VILK for use by the poultry industry.

Purification of the effector cytokine and/or isolation of the gene(s) will lead to the licensing of this technology by Eli Lilly and Company.

OBJECTIVE B: Develop a *Salmonella*-immune lymphokine (PILK) to control *Salmonella* infections in swine.

PROGRESS B: A PILK has been developed by immunizing swine with *S. enteritidis*. The PILK has been tested in a collaborative effort with Dr. Edward McGruder at Eli Lilly and Company. In preliminary experiments, dramatic results have been observed in both pathogeneses and food safety experiments. Oral administration of as little as 10μg of PILK/pound eliminate the morbidity and mortality of a *S. cholerasuis* (10^9 cfu) challenge in 14-day-old swine. Additionally, oral administration of PILK protected weaned pigs against organ invasion by *S. cholerasuis* and significantly reduced intestinal colonization by *S. cholerasuis* in these pigs.

IMPACT/TECH TRANSFER B: A second CRADA with Eli Lilly and Company on PILK control of swine infectious diseases is being developed. The successful development of a PILK will provide swine producers a new technology to control salmonellosis and other infectious agents in swine.

OBJECTIVE C: Evaluate the effects of immune-lymphokines on resistance to *Listeria monocytogenes* in newly hatched chickens.

PROGRESS C: The consequences of oral, intracloacal, and intraperitoneal challenge with *Listeria monocytogenes* on morbidity, cecal colonization, and organ invasion were evaluated. While it was found that morbidity occurred when 4 day old chicks were challenged by the i.p. route with *Listeria monocytogenes*, no morbidity or mortality occurred following oral or intracloacal challenge, and no mortality occurred when 4 day old chicks were challenged by the i.p. route. Organ invasion is minimal and *Listeria monocytogenes* is seldom recovered from the livers or spleens of challenged birds. Cecal colonization occurs by either the oral or intracloacal route and can be controlled by oral treatment with competitive exclusion cultures. Because *Listeria monocytogenes* was seldom recovered from the livers of challenged birds, it appears to be a poor candidate for control by the SILK cytokine.

IMPACT/TECH TRANSFER C: This information suggests that competitive exclusion cultures used to control other enteropathogens will have a secondary benefit in terms of control of *Listeria monocytogenes.*

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PATHOGENESIS, DETECTION, AND CONTROL OF *SALMONELLA ENTERITIDIS* AND OTHER SALMONELLAE IN CHICKENS

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OBJECTIVE A: Produce a strain of *Salmonella enteritidis* (SE) that grows to higher cell density while maintaining production of O-chain lipopolysaccharide (O-chain LPS)

PROGRESS A: The ability of bacteria to grow to high cell densities is a shared characteristic used by nearly all plant and animal pathogens to achieve a high rate of infection, or penetrance, in host populations. However, numerous evaluations for high cell density growth of *Salmonella* with genetic reporter systems have failed to indicate that this highly successful pathogen uses this common virulence strategy. Rather than concluding that an important virulence strategy was absent from *Salmonella*, investigations were begun on factors that limited high cell density growth. Previously, virulent *Salmonella* serovar Enteritidis (SE) was reported to lose its ability to produce O-chain LPS if growth exceeds 2.9 x 10^9 colony forming units per ml (cfu/ml), even though it could continue to grow to 3.8 x 10^9 cfu/ml. Growth curves conducted with highly virulent SE indicated that it could grow to 3.9 x 10^9 cfu/ml while maintaining production of O-chain LPS. Since strains that lack O-chain are avirulent, it became important to isolate a stable strain of SE that could grow to high cell density while maintaining production of O-chain LPS. A strain with the desired characteristics, SE-HCD, was constructed and isolated, and current evaluations indicate that it can grow to 9 x 10^9 cfu/ml while maintaining production of O-chain LPS. SE-HCD is exquisitely sensitive to environmental conditions, and responds to changes in signals by increasing or decreasing transcription of the *lux*R regulated gene family. These results suggest that stringent regulation of growth to high cell density is an important part of the basic biology of SE.

IMPACT/TECH TRANSFER A: Although poultry vaccines against SE are somewhat effective, vaccination failures occur. The SE-HCD strain was developed as part of a long-range, high risk approach to provide to industry a challenge model that would stringently assess the ability of killed and attenuated live vaccines to prevent egg contamination. This work was supported by CRADA No. 58-3K95-5-364.

OBJECTIVE B: Identify outer membrane characteristics of phage type 4 *Salmonella* serovar Enteritidis that are different from those of other SE phage types.

PROGRESS B: Certain phage types (PTs) of *Salmonella* serovar Enteritidis (SE) are routinely isolated from contaminated eggs, the hen-house environment, rodents, and from infected people.

In the U.S., these PTs included PTs 8, 13a, 23, 13 and untypable strains. Within the past three years, PT 4 has also been isolated in the U.S., which is a notable change in U.S. epidemiology since PT 4 has been historically associated with increased incidence of illness in people in other countries worldwide. Only two laboratories in the U.S., the Centers for Disease Control (Atlanta, Georgia) and the National Veterinary Services Lab (Ames, Iowa), classify SE using typing phages, which limits our ability to conduct routine surveillance for the emergence of PT 4 SE. In order to devise techniques that would allow anyone who cultures SE to evaluate every isolate for being PT 4, the outer membrane characteristics unique to PT 4 SE were characterized. A specialized inducing media indicated PT 4 SE rarely produces H2 flagellar epitopes, whereas all other phage types alter H-antigen immunoreactivity from that accepted as standard for SE. Other assays indicated that PT 4 SE exhibited the same type of O-chain LPS variation and growth properties as other SE PTs, and that it is common to encounter virulent and avirulent PT 4.

IMPACT/TECH TRANSFER B: This assay is available for immediate use by FSIS and diagnostic laboratories for the purpose of inexpensively grouping all SE isolates into PT 4 and non-PT 4 lineages. The specialized growth media has a patent pending, and is currently available for licensing.

OBJECTIVE C: Determine the role that rodents have in propagating *Salmonella enteritidis* (SE) in the hen-house environment.

PROGRESS C: A collaborative project between ARS and APHIS, with some research funding provided by NRICGP, determined that the house mouse, *mus musculus*, is an important reservoir of invasive SE. Of 621 and 526 spleens cultured during a first and second year of collection, 25.0 and 17.9%, respectively, were positive for SE. Contaminated eggs were cultured from nine houses during the first year of sampling, and for eight of these houses, SE was recovered from the spleens of mice. Rank sum statistical analysis of positive mouse spleens indicated that three overlapping bacterial populations were present. This pattern of infection was repeated when lipopolysaccharide (LPS) variants were used to infect chicks, and the worst infections were associated with isolates producing a high-molecular-weight (HMW) LPS. Mouse isolates were capable of producing unprecedented amounts of HMW-LPS as indicated by compositional analysis of six isolates that swarmed across 2% agar. It is suggested that monitoring serovar Enteritidis cultured from the spleens of mice caught on farms will detect the emergence of SE and the presence of strains that are enhanced in their ability to contaminate eggs.

IMPACT/TECH TRANSFER C: Culturing mouse spleens provided as much information about the presence of SE in hen-houses as did environmental sampling. In addition, culturing spleens simplified culture methodology and provided information about the presence of virulent SE on farms. An immediate finding of this work is that colanic acid positive phage untypable strains of SE originate from hyperflagellating, swarming isolates that produce HMW-LPS.

OBJECTIVE D: Examine the development of serum and intestinal immunity against *Salmonella enteritidis* (SE) in chickens infected with SE at 1 day of age.

PROGRESS D: Infection in the hatchery is an important site for entry of SE into a flock. Little information is available regarding how well birds infected at this early age can elicit a systemic and an intestinal mucosal immune response against SE. We found that birds infected with SE at 1 day of age mounted a poor immune response to the challenge and this hyporesponsiveness did not improve as the birds matured and went into egg lay 20-23 weeks postchallenge. On many sampling days 50% of birds which were found to be organ positive for SE did not produce a detectable serum or intestinal response. Further, some birds were found to be intestinally shedding 10^3-10^4 SE/gram of feces in spite of a good mucosal immune response, indicating the inability of the immune response to eliminate a chronic, well-established infection.

IMPACT/TECH TRANSFER D: As serology is an important component of many egg quality assurance programs, these data show that birds infected with SE at such an early age can have a significant effect on the ability of this testing scheme to detect SE-infected flocks. Special attention therefore needs to be directed at preventing exposure of chicks to this pathogen in the hatchery and at screening the breeder flocks for potential SE problems.

OBJECTIVE E: Develop and evaluate rapid and sensitive methods for detecting *Salmonella enteritidis* (SE) infection in chickens and contamination in egg contents.

PROGRESS E: Quality assurance programs for reducing egg-associated transmission of SE to humans often rely on culturing pools of egg contents to detect infected laying flocks. After egg contents are pooled together for sampling, they are often incubated to allow small numbers of SE cells to multiply to easily detectable levels. The direct plating method for culturing eggs relies on this incubation step to increase SE levels so that a sample of each pool can be transferred directly to agar culture media for detection of SE (without any additional enrichment steps). Direct plating is simple and rapid, but may not detect very small numbers of SE. The present study evaluated the effectiveness of adding highly concentrated broth enrichment media to incubating egg pools for promoting SE growth and thereby supporting detection by direct plating. When pools of liquid whole egg were contaminated with fewer than 10 SE cells each, both iron and concentrated media supplementation improved SE recovery. Supplementation with concentrated tryptone soya broth resulted in significantly better SE recovery than did iron supplementation. Highly efficient presumptive detection of very low incidences and levels of SE contamination by direct plating was accomplished in a total of 48 hours by adding tryptone soya broth (at 5 times the normal concentration) to incubating egg pools.

Salmonella enteritidis (SE) contamination of eggs is a significant cause of human illness. Testing to detect SE infections in laying hens is an important component in efforts to reduce the likelihood that contaminated eggs will reach consumers. In such testing programs, a preliminary screening test is usually applied to select flocks for subsequent confirmatory testing. Testing for specific antibodies in egg yolks has previously been shown to be capable of effectively predicting the probability of egg contamination, but the sensitivity of this type of screening test has not been established. In the present study, groups of hens were experimentally infected with various doses of SE (ranging from 10^3 to 10^7) and eggs were tested for antibodies to SE flagella using an enzyme immunoassay. All hens that were inoculated with 10^7 cells of SE were detected as infected by the egg yolk ELISA, as were up to 66% and 35% of hens inoculated with 10^5 or

10^3 cells, respectively. Even when yolks from infected hens were diluted 1:10 in yolk from uninfected hens (simulating the pooling together of 10 eggs for sampling), specific antibodies could still be found in eggs from as many as 31% of hens given 10^7 cells of SE and 13% of hens given 10^3 cells.

IMPACT/TECH TRANSFER E: This research provided regulatory officials, diagnostic laboratories, and the poultry industry with a rapid and sensitive method for detecting small numbers of SE contaminants in egg contents and demonstrated that egg yolk antibody testing can be applied to individual or pooled samples to achieve rapid and sensitive screening for SE infection in laying flocks.

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CONTROL OF *SALMONELLA* IN DOMESTIC ANIMALS

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OBJECTIVE A: Develop control procedures to prevent colonization of chickens and/or turkeys with competitive exclusion cultures.

PROGRESS A: Under a Cooperative Research and Development Agreement with the Continental Grain Company, our patented mucosal competitive exclusion (MCE) culture is undergoing final FDA, NADA trials. Dose titration and target animal safety studies have been completed and submitted to FDA for final approval. Three field trials in commercial production facilities to demonstrate efficacy of the licensed MCE product have been successfully completed and the data is currently being compiled for submission to FDA.

IMPACT/TECH TRANSFER A: Two patents have been issued and licensed by commercial companies. The MCE patent has been licensed by Continental Grain Company and they anticipate having an FDA approved product on the market in the first six months of 1998. *In ovo* application of bacterial cultures has been licensed by the EMBREX company. A new CRADA with the Continental Grain Company has been developed to study the use of our competitive exclusion technology to control *Salmonella* in porcine and bovine.

OBJECTIVE B: Develop control procedures to prevent colonization of chickens and/or turkeys with the yeast *Saccharomyces boulardii*.

PROGRESS B: We have been investigating the ability of the yeast, *Saccharomyces boulardii*, to reduce *Salmonella* colonization of poultry when administered in the egg *(in ovo)* or in the feed. Recent experiments have been designed to determine the most practical yeast delivery methods. Studies have shown no adverse effect of administering the yeast concomitantly with Marek's vaccine which is commonly delivered *in ovo*. Feeding trials have demonstrated effect of mannan (yeast cell wall product) as well as viable yeast. If current floor pen trials confirm these results, field trials of the feed treatments should begin within the next 6 months.

IMPACT/TECH TRANSFER B: Two patents have been written, a Cooperative Research and Development Agreement is in effect with the Lallemand company who has licensed this patent pending technology. There is significant potential impact for this organism, that is approved for use to treat humans with antibiotic associated diarrhea, to greatly assist in controlling the initiation and persistence of salmonellae colonization of the chicken intestinal tract.

OBJECTIVE C: Develop and/or evaluate intervention measures for reducing *Clostridium perfringens (Cp)* in poultry

PROGRESS C: The age of susceptibility of broiler chicks to *Cp* proliferation and the intestinal loci for colonization were determined. Numbers of *Cp* were low ($<\log_{10}$ 4.0/g) in all areas of the intestinal tract and in feces of chicks challenged with *Cp* and maintained on corn-based feed for 21 days. For chicks given a 50 %-rye diet, *Cp* numbers were low during the first 2-days post-challenge, but increased to $\log_{10}$ 6.9 by day 7 and $\log_{10}$ 8.6 by day 21, numbers of *Cp* found in birds suffering from necrotic enteritis. Of seven intestinal sites sampled, the greatest *Cp* numbers were found in the ileum and the ceca. The MCE, a yeast culture, and a defined Competitive Exclusion culture when given chicks in controlled experiments reduced subsequent numbers of *Cp* and enterotoxin levels in the ceca of rye-fed birds.

IMPACT/TECH TRANSFER C: *Cp* proliferation in the poultry intestinal tract is a factor contributing to necrotic enteritis in poultry and, by spread through the poultry production environment, contributing to contamination of poultry used for human consumption. Information about the dynamics of *Cp* colonization provides knowledge to determine the best times and sampling sites for measuring *Cp* levels in a poultry flock. Determination of *Cp* levels could alert the producer to the need for therapeutic measures to prevent disease associated with *Cp*. These determinations also indicate the effectiveness of intervention procedures, i.e., competitive exclusion cultures, in reducing the intestinal populations of *Cp*. Preventing *Cp* proliferation reduces the liklihood of poultry disease associated with *Cp* and human disease resulting from consumption of *Cp*-contaminated poultry.

OBJECTIVE D: Determine the sources of salmonellae and develop epidemiological risk assessment models for salmonellae during poultry production.

PROGRESS D: A protocol has been developed and initiated to identify most frequent sources of salmonellae, *Campylobacter* and *Clostridium perfringens* during poultry production and to determine which of these sources contribute most frequently to the types of these pathogens found on final processed carcasses. In five commercial field trials involving over 250,000 birds, it was shown that when hatchery contamination was less than 5% the prevalence of salmonellae on processed carcasses was less than 20% and when hatchery contamination was greater than 15% then prevalence of salmonellae on processed carcasses was greater than 25%.

IMPACT/TECH TRANSFER D: Epidemiological data on sources and spread of salmonellae and other pathogens in poultry production is needed in order to develop effective risk assessment models for these pathogens. Developing a more complete knowledge of the principal sources of these pathogens will allow researchers to better focus research and the poultry industry to better focus resources for control during poultry production.

OBJECTIVE E: Determine the role of the outer egg shell membrane in controlling penetration of salmonellae into the fertile hatching egg.

PROGRESS E: Using confocal microscopy, the outer shell membrane of fertile hatching eggs was studied over the full production cycle of a commercial breeder flock. No changes in the open spaces of the outer shell membrane were observed as the breeder flock aged and the eggs got larger. No apparent relationship was observed in the propensity of salmonellae to penetrate outer shell membranes and the amount of open spaces in the membranes.

IMPACT/TECH TRANSFER E: These data further confirm that no matter the age of the breeder flock, eggs need to be sanitized before salmonellae has an opportunity to penetrate the eggs shell and related membrane structure. Once salmonellae penetrate the egg shell membranes, it is very difficult to get chemical sanitizers into contact with the salmonellae.

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EPIDEMIOLOGY AND CONTROL OF *SALMONELLA*

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CRIS Number: **3625-32000-029**
FSIS Code: **N1AM01**
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OBJECTIVE A: To identify virulence factors important in the pathogenesis of *S. choleraesuis* and *S. typhimurium* in swine.

PROGRESS A: A study was conducted to investigate the effects of exposure to porcine reproductive and respiratory syndrome (PRRS) virus, *S. choleraesuis*, and stress on young pigs. Results support field observations that clinical outbreaks of PRRS virus are the result of interactions among concurrent infections and stressors. Serum levels of interferon-gamma (IFN) decreased after PRRS virus exposure, but not after *S. choleraesuis* exposure or administration of the stressor. The addition of stress following *Salmonella* exposure caused a dramatic decrease in serum IFN levels, which may indicate a state of immunosuppression and increased susceptibility to PRRS virus infection.

IMPACT/TECH TRANSFER A: Until recently, experimental infection with PRRS virus did not lead to the severe clinical signs associated with PRRS virus infection in the field. This work further defines a reproducible experimental model that replicates clinical signs in the field associated with PRRS virus respiratory disease and concurrent bacterial infection, in this case *S. choleraesuis*. The effect of disease on cell-mediated immunity was further defined. This work is an important first step for researchers interested in developing vaccines against PRRS virus and associated pathogens such as *Salmonella*. Results have been presented to commodity groups and other professionals at scientific meetings.

OBJECTIVE B: To define the epidemiology and transmission of *Salmonella* in swine.

PROGRESS B: *Salmonella heidelberg* is a nonhost-adapted serotype of swine, the fourth most frequently isolated *Salmonella* serotype from swine and humans. Various aspects regarding the pathogenesis, carrier state, or transmission of *Salmonella heidelberg* are relatively unknown. The effect of inoculation dose on shedding of *S. heidelberg* was studied. Pigs received 10^3, 10^6, or 10^9 cfu (colony forming unit) *Salmonella heidelberg*. Inoculation with *S. heidelberg* did not result in apparent clinical disease, regardless of dose. However, a dose dependent correlation was observed with respect to shedding and number of positive tissues. In a separate study, the effect of two antimicrobics, Naxel™ (ceftiofur) and Baytril™ (enrofloxacin), on *S. heidelberg* infection in swine was studied. No increase in resistance was observed for any isolate obtained

throughout the six-week study. Baytril™ appeared to be more effective than Naxcel™ in reducing *Salmonella* numbers.

IMPACT/TECH TRANSFER B: The dose and persistence studies have furthered our knowledge regarding the carrier state of *Salmonella* in swine. This information will be useful to producers, practicing veterinarians, and scientists in related areas. The antibiotic studies will be of interest to producers, practicing veterinarians, federal action agencies (APHIS, FSIS, FDA), and the feed industry. This work has been presented to commodity groups and other professionals at scientific meetings.

OBJECTIVE C: To define the porcine immune response to acute and chronic *Salmonella* infection focusing on mechanisms to reduce or eliminate the pathogenic organism.

PROGRESS C: Modulation of the cytokine, tumor necrosis factor (TNF), or its natural inhibitor, tumor necrosis factor receptor-1 (TNFR-1), may offer a means to prevent the harmful events associated with *S. choleraesuis*-induced septicemia and the spread of *Salmonella* during marketing of swine. Recombinant porcine TNFR-1 has been expressed and partially purified and used to produce monoclonal antibodies. We are currently developing an ELISA assay using these monoclonal antibodies to measure levels of TNFR-1 in pig blood. Patterns of TNF and TNFR-1 may be of diagnostic or prognostic significance in salmonellosis; however, the interactive nature of receptor and cytokine must be determined before a full understanding of mobilization of porcine defenses against salmonellosis and other pathogens is possible.

Research to determine swine resistance to *Salmonella* infection based on genetic background has begun. An important first step is to develop a screening system/assay able to identify *Salmonella* in "susceptible" and "nonsusceptible" animals. We are currently working on an *in vitro* macrophage bactericidal assay using flow cytometry. Genetic control of disease resistance using selective breeding programs has become a viable proposition for disease control. By studying porcine mechanisms of defense against *Salmonella* at the molecular level it will be possible to discover new therapeutic avenues for intervention against salmonellosis and other diseases.

IMPACT/TECH TRANSFER C: A better understanding of porcine cytokine responses to intracellular pathogens such as *Salmonella* will lead to more rational approaches in vaccine design and the use of recombinant cytokines as immunological adjuvants or immunomodulators. Veterinary biologic producers, breeders, and scientists in related areas will benefit from the information and products generated from this research.

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NATIONAL ANTIMICROBIAL SUSCEPTIBILITY MONITORING

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Historical: In early 1995 ARS and APHIS began a study to assess the degree of resistance among *Salmonella* isolates of veterinary origin. In late 1995, the FDA-CVM initiated a surveillance program involving both the ARS/APHIS and CDC, which was starting a similar monitoring program for human pathogens. It was determined that ARS/APHIS would monitor the change in susceptibility over time in veterinary isolates while the CDC would monitor human isolates. *Salmonella* was chosen as the sentinel organism because it is an important foodborne pathogen, it is ubiquitous in nature, it is reportable for CDC, and both human and veterinary isolates are available. This program has come to be known as the National Antimicrobial Susceptibility Monitoring System.

PROGRESS A: In 1996 1,041 *Salmonella* isolates of veterinary origin were tested against 16 antimicrobics. Isolates were obtained from cattle, swine, chickens, turkeys, swine feed, ground product, exotics, dogs, and cats from both clinical and nonclinical isolations. All isolates were susceptible to amikacin, cepotaxime, and ciprofloxacin. Approximately 34% and 28% of the isolates were resistant to tetracycline and sulfamethoxazole, respectively, while 13% of the isolates were resistant to both ampicillin and ticarcillin. Less than 9% resistance was observed for all other antimicrobics.

IMPACT/TECH TRANSFER A: Development of resistance to antimicrobics is a major concern in both human and animal medicine. With the limited availability of new drugs to combat pathogens, prudent and judicious use of antimicrobics is warranted. These data will serve as baseline values for comparison of future studies and provide important information to the veterinary and medical community as to the development of resistance.

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DEVELOPMENT OF COST EFFECTIVE MEANS TO PREVENT AND CONTROL SALMONELLOSIS IN POULTRY

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OBJECTIVE A: Develop a cost effective means to prevent/control salmonellosis in poultry.

PROGRESS A: During the past year, our CRADA partner, Milk Specialties Company, Dundee, IL, completed plant facilities and secured FDA approval for the commercial production of our USDA patented defined CF3™ competitive exclusion culture. The final FDA pivotal field studies to evaluate CF3™ control of *Salmonella* on commercial broiler farms were completed. The BioScience Division of Milk Specialties Company is presently preparing the final report of the field study results for submission to FDA to obtain registration approval and commercialization of the CF3™ culture.

IMPACT/TECH TRANSFER A: The patent for the defined CF3™ culture was issued on 12/26/95, and the patent for the continuous-flow process of selecting and maintaining the culture was issued 2/18/97. During the past year, Milk Specialties Company has produced 100 million doses of the CF3™ culture for treatment of poultry in Japan contracted by sub-licensee Kyoritsu Shoji Company, Tokyo, Japan. Additionally, Milk Specialties has completed agreements with ELANCO (Eli Lilly Co.) for international distribution of CF3™ upon receiving FDA approval. The ARS inventor of CF3™ received the USDA-ARS Technology Transfer Award (12/11/96) and the Federal Laboratory Consortium Award for Excellence in Technology Transfer (4/15/97), and CF3™ was used as an example of ARS technology in the recent Congressional Budget Hearings.

OBJECTIVE B: Evaluate the chicken crop as a source of *Salmonella* and *Campylobacter* contamination of poultry slaughter plants.

PROGRESS B: Feed is withdrawn from broiler chickens 6 to 12 hours before transport to the processing plant. During feed withdrawal, the birds increasingly peck at fecal droppings and litter on the rearing house floor. We recently demonstrated that *Salmonella* contamination of the crop increases dramatically during this feed withdrawal period. Additionally, we found that the percentage of crops that are ruptured during removal in the processing plant was as high as 25%. These results suggest that contaminated crops may result in the contamination of processing equipment and serve as a major source of carcass contamination.

IMPACT/TECH TRANSFER B: The feed withdrawal period, immediately prior to broiler processing, may represent a preharvest critical control point. The identification of sources of *Salmonella* that enter the processing plant permits industry and researchers to develop effective intervention strategies to reduce the number of human pathogens that enter processing facilities.

OBJECTIVE C: Determine the mechanism by which forced molt, induced by feed withdrawal, decreased the resistance of laying hens to *Salmonella enteritidis* (SE) infection and further determine the mechanism by which the addition of lactose to the drinking water of molting hens reestablishes resistance.

PROGRESS C: Molting induced by feed withdrawing is used by the layer industry to stimulate egg laying in aging flocks of hens. Research has clearly demonstrated that feed removal and molting decreases the resistance of hens to SE infection resulting in severe infections, increased fecal shedding of SE, and the rapid spread of infection to molted hens in neighboring cages. The mechanism by which forced molt decreases resistance to SE infection is unknown. We recently demonstrated that the addition of lactose (milk sugar) to the drinking water of hens during molting restores normal resistance to SE infection. The mechanism of lactose restored resistance is unclear and requires clarification to permit practical application to the layer industry.

IMPACT/TECH TRANSFER C: Sixty percent of the approximately 300 million laying hens nationwide are forced molted placing nearly 180 million hens at increased risk to SE infection. There is clearly a need to investigate the mechanism(s) by which feed withdrawal and forced molt reduces resistance to SE and to identify prophylactic or therapeutic measures such as the use of lactose that will maintain or reestablish resistance.

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CONTROL OF *SALMONELLA* AND *ESCHERICHIA COLI* O157:H7 IN LIVESTOCK DURING THE PREHARVEST PERIOD

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OBJECTIVE A: To develop rapid diagnostic tests for *E. coli* O157:H7.

PROGRESS A: A simple, highly specific test for the detection of *E. coli* O157:H7 has been developed. The reagents developed for this test consist of two monoclonal antibodies, MARC13B3 and MARC19F8, derived using standard methods from mice immunized with *E. coli* O157:H7 CDC EDL933. These two monoclonal antibodies have been formatted as a lateral diffusion immunocapture assay by Meridian Diagnostics, Inc., utilizing proprietary antibody coating chemistry. The test is highly specific for pathogenic *E. coli* O157:H7 and O157:NM, eliminating false positives caused by other, non-enterohemorrhagic *E. coli* O157 types, group N *Salmonella*, *Yersinia enterocolitica* O9, and other cross-reacting bacteria.

IMPACT/TECH TRANSFER A: This technology has been transferred to a U.S. corporation, Meridian Diagnostics, Inc., and is currently being marketed to the meat industry. The test is tentatively designated ImmunoCard Stat! *E. coli* O157:H7 by Meridian. The simplicity and specificity of the test should result in widespread use by the meat industry.

OBJECTIVE B: To develop rapid tests for Salmonellae in livestock.

PROGRESS B: Monoclonal antibodies specific for the six most common serogroups of *Salmonella*, B, C1, C2-3, D1, E1, and E4, have been developed. Serotype specific monoclonals for *Salmonella typhimurium*, *S. typhimurium copenhagen*, *S. dublin*, and *S. choleraesuis* have also been developed. This set of antibodies will detect greater than 90% of the *Salmonella* serotypes isolated from livestock and humans in recent years. They have been utilized in an ELISA format and will be formatted into a simplified test device in the near future.

IMPACT/TECH TRANSFER B: Numerous inquiries from U.S. and overseas companies have been received regarding the potential use of these antibodies in diagnostic tests for meat, poultry, seafood, and live animals. We anticipate initiation of a competition for use of these antibodies this year.

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PREVENTION OF LOSSES FROM COLIBACILLOSIS AND *ESCHERICHIA COLI* O157:H7 IN CATTLE AND SWINE

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OBJECTIVE A: Identify site and mechanism of *E. coli* O157:H7 colonization in cattle and identify other virulence attributes of O157:H7 and Shiga toxin-producing *E. coli.*

PROGRESS A: *E. coli* O157:H7 and other serotypes of Shiga toxin-producing *E. coli* (STEC) that cause human disease have been isolated from cattle. These bacteria colonize the intestinal tract and are shed in feces of cattle. This can result in contamination of beef and dairy products, resulting in foodborne outbreaks. Reducing the amount of O157:H7 in the intestinal tract and feces of cattle should decrease the incidence of human disease linked to the consumption of beef and dairy products. In order to reach this goal, we must first determine where and how O157:H7 and other STEC grow and survive in the intestine of cattle.

We have previously shown that newborn calves are very susceptible to experimental infection with O157:H7 and frequently develop severe diarrhea. Large numbers of O157:H7 are found in the large intestine, ileum, and feces of newborn calves inoculated with the bacteria. The O157:H7 attach to and damage the intestinal surface. We have now determined that while inoculation of weaned calves with O157:H7 does not result in disease, the bacteria attach to and damage the large intestine in 3 of 7 weaned calves. These experiments in weaned calves provide the first insight into how and where O157:H7 colonizes the intestine of older cattle.

STEC and O157:H7 that cause human disease produce Shiga toxins, contain a large 60 megadalton plasmid and usually contain the *eae* gene which encodes intimin (a bacterial protein involved in bacterial attachment to intestinal cells). We inoculated animals with O157:H7 strains that were no longer able to produce either Shiga toxins or intimin and compared these results to inoculation with wild-type O157:H7 which produced both of these factors. Strains that did not produce either intimin or Shiga toxin were found in lower numbers in the intestines and feces of weaned calves than was the wild-type O157:H7 strain. Results in newborn calves inoculated with the same strains gave different results. Intimin was found to have a role in both colonization of the intestine and disease in newborn calves. However, the strain that did not produce Shiga toxin colonized as well as wild-type O157:H7 and the severity of disease was similar. This work identified two important bacterial virulence factors that have a role in O157:H7 colonization in older cattle, one of which, intimin, also has a role in newborn calves.

This demonstrates that both intimin and Shiga toxin have a role in colonization in weaned calves but that, of these, only intimin is required for pathogenesis in newborn calves.

IMPACT/TECH TRANSFER A: These results provide insight into O157:H7 virulence factors required for colonization of cattle. Therapies that target intimin or Shiga toxin may prevent or reduce O157:H7 and other STEC infections in cattle. This work was a major reason why the USDA has funded a collaborative study with Dr. A. O'Brien, Bethesda, MD, and other investigators. In these studies, we will attempt to reduce shedding of O157:H7 in animals by delivering anti-intimin antibodies to the intestine. Dr. Mark Ackermann is using the neonatal calf O157:H7 infection model to determine the role neutrophils play in intestine damage due to O157:H7 infections. Also, feces from our experimentally inoculated animals have been used by scientists at the Meat Animal Research Center (MARC) to develop methods for reducing O157:H7 contamination of beef carcasses by spray washings.

OBJECTIVE B: Identify methods to reduce shedding of *E. coli* O157:H7 in cattle.

PROGRESS B: A promising approach for reducing the level of O157:H7 in cattle is to stimulate a protective intestinal immune response. Theoretically, the best method of stimulating a protective immune response is oral vaccination with a live strain. However, the vaccine must be safe and avirulent so that the vaccine strain itself is not a potential foodborne pathogen.

We have tested an O157:H7 strain that no longer produces Shiga toxin (see objective A) as a potential vaccine for O157:H7 in cattle. Three groups of weaned calves were used in this experiment. One group was orally "vaccinated" twice with the potential vaccine strain, one group with a wild-type O157:H7 strain that produced Shiga toxin, and one group with a nonpathogenic control strain. All groups were then challenged with a wild-type O157:H7 strain. All three groups shed similar amounts of wild-type O157:H7 after challenge demonstrating that the vaccine did not reduce shedding. However, the toxin-negative vaccine strain induced a broader immune response in weaned calves (both antibody and cellular) than did the wild-type O157:H7 strain (only an antibody response). This suggests that the toxin is immunosuppressive in cattle.

In a preliminary attempt to counteract the immunosuppressive effects of toxin in cattle, we parenterally vaccinated calves with a genetically altered form of Shiga toxin. This mutant toxin is a million-fold less toxic to animals than is wild-type toxin. This mutant toxin was used to vaccinate two calves and did not reduce shedding of wild-type O157:H7. Interestingly, this mutant toxin did affect shedding of the toxin-negative O157:H7 strain (see Objective A). Two calves vaccinated with the mutant toxin and then orally inoculated with the toxin-negative strain shed higher numbers of the inoculum strain than did nonvaccinated calves.

The higher fecal shedding of the toxin-positive O157:H7 strain relative to the toxin-negative strain (Objective A) and increased shedding of the toxin-negative strain in weaned calves vaccinated with mutant toxin demonstrate that both toxin and mutant toxin affect colonization.

Whether this effect is due to immunosuppression or due to some other toxin-induced effects remains to be determined.

IMPACT/TECH TRANSFER B: Vaccinating cattle for O157:H7 may be possible, but scientists and vaccine companies need to consider that Shiga toxins may be immunosuppressive. Immunoassays developed by scientists and diagnostic laboratories for identifying cattle exposed to O157:H7 should be antibody based, not cellular based. In addition, it is possible that the immunosuppressive properties of the toxin may make cattle more susceptible to other pathogens, including zoonotic ones. Sera from these vaccinated animals have been shared with investigators at MARC who are developing a diagnostic test to identify O157:H7-infected animals and researchers at University of Maryland who are studying secretion proteins of O157:H7 (espB).

OBJECTIVE C: Develop rapid methods to identify and quantify *E. coli* O157:H7 and other STEC pathogenic for humans in tissues and fluids from cattle.

PROGRESS C: As described in Objective A, newborn calves develop severe diarrhea after experimental infection with STEC. Other *E. coli* types (enterotoxigenic and enteropathogenic), which are not considered pathogenic for humans, also cause diarrhea in calves but the relative prevalence of these three types in sick calves is not known. In collaboration with scientists at Iowa State University, we have developed a rapid, gene-based assay (multiplex PCR) to identify which type of *E. coli* is associated with diarrhea in calves.

IMPACT/TECH TRANSFER C: Newborn calves with diarrhea on the farm are a potential source of O157:H7 and other STEC. Past studies have demonstrated that 1% to 2% of older calves (> 3 weeks) shed O157:H7, but the incidence of O157:H7 and other STEC in newborn calves (< 3 weeks) with diarrhea is unknown and needs to be determined. These newborn calves could infect producers and veterinarians directly and be a source of infection for older cattle on the farm. Prompt treatment or removal of these animals from the herd will decrease the direct risk to producers and veterinarians and reduce the prevalence of O157:H7 and other STEC in the herd. This herd reduction might decrease the level of STEC in cattle as they enter the slaughter plant and positively impact the safety of beef and dairy products.

Our multiplex PCR assay allows rapid identification and classification of *E. coli* associated with calf diarrhea with a single test. As this assay can identify STEC, enterotoxigenic and enteropathogenic *E. coli,* the results would be more informative for diagnostic labs, producers, and veterinarians than an assay specific for O157:H7 or STEC.

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CONTROL OF *CAMPYLOBACTER JEJUNI* IN POULTRY

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OBJECTIVE A: Determine the optimum transport medium for enumerating *Campylobacter* spp., *E. coli*, and *Salmonella typhimurium* from broiler carcass rinses after 24 hour transport on artificial ice.

PROGRESS A: One hundred carcasses were obtained from 2 local processing plants and were analyzed by the FSIS and ARS laboratories. Each carcass was rinsed per FSIS protocol with 400 ml sterile water and 90 ml were decanted into each of 3 separate sterile containers. To these rinses 10 ml of either a) sterile tap water; b) 10X buffered peptone water (BPW); or c) 10X universal pre-enrichment (UP) broth were added. We spiked each of the rinses with an antibiotic resistant strain of chicken passaged *S. typhimurium* (10^3/ml). After initial microbial analyses, samples were packed into insulated coolers and were kept cold with artificial ice packs for ca. 24 hours, when analyses was repeated. Variation between the two laboratories was statistically non-significant for levels of *Campylobacter* spp., *E. coli*, and *Salmonella typhimurium*. Overall, there was an insignifcant loss ($p>0.05$) in cell numbers during the 24 hour simulated transport for each of the species sampled. There was very limited difference between levels of the three target bacteria detected by using either tap water or BPW as transport medium. In a subsequent study, we determined that the variation in pH (6.5-7.5) and water hardness (36 to 136 ppm $CaCO_3$) seen in potable waters in the United States did not significantly affect the ability to recover campylobacters from carcass rinses before or after shipping.

IMPACT/TECH TRANSFER A: The FSIS immediately began applying these findings to ship chilled BPW rinse samples from processing plants around the United States to their microbiology laboratories for further analyses of *E. coli* and salmonellae.

OBJECTIVE B: To evaluate microbiological techniques for rapid, cost effective enumeration of *Campylobacter* spp. in poultry samples.

PROGRESS B: Experiments were conducted to compare enumeration of *Campylobacter* from fully processed broiler carcasses by three techniques; a most probable number (MPN) method using Hunt's enrichment broth (HEB) and *Campylobacter* charcoal differential media (CCDA); and MPN method using Rosef's enrichment broth (REB) and Mueller-Hinton blood agar; and a direct plating method using Campy-Cefex agar. Significantly fewer campylobacters were recovered from carcasses by the MPN method using REB than the other two methods.

Estimations of *Campylobacter* populations recovered from the broiler carcasses were not significantly different between the direct plating method and the MPN method utilizing HEB and CCDA. The direct plating method was much simpler to perform than the MPN and represents a significant time and cost savings.

IMPACT/TECH TRANSFER B: This information is directly applicable to sampling procedures used by regulatory agencies. This will allow accurate estimations of *Campylobacter* populations associated with greater numbers of broiler carcasses and reduce time and cost.

OBJECTIVE C: Develop a vaccine that prevents *Campylobacter* colonization of chickens.

PROGRESS C: We have been testing a vaccine designed for oral use to immunize chickens against *C. jejuni*. The vaccine is a genetically engineered fusion of the binding subunit of LT toxin (LT-B) of *Escherichia coli* with a portion of the flagellin gene (fla) from *C. jejuni*. LT-B is a mucosal adjuvant in that it is known to increase secretory immune response against antigens given orally together with the reagent. This activity is dependent on the LT-B binding to G_{M1} ganglioside that is a part of the membrane of the host cells. To evaluate the results we developed an assay to measure the antibody response to components of the *Campylobacter*. The assay is a semi-quantitative Western blot in which we can evaluate the response against several antigens. We have applied the assay to serum and fecal antibodies. There was no correlation of serum antibodies with fecal antibodies or with levels of *Campylobacter* found in the bird. A new form of the vaccine has been constructed that is composed of portions of the flagellin that are highly conserved through the *Campylobacter* species. Higher yields of the vaccine protein have been produced with the new vaccine. We are currently evaluating the new vaccine and also evaluating the response to the vaccine in the presence of toxins that interupt with the ganglioside binding site. We have also analyzed the chicken cellular immune response to Campylobacter and found that extracts from the organism cause a non-specific induction of T-cell death. This may represent a mechanism that the organism uses to escape immunity. Capitalization on this discovery will help in design of new vaccine formulations.

IMPACT/TECH TRANSFER C: A Confidentiality Agreement has been instituted with Intervet, Inc., for the purpose of allowing the company to test the feasibility of a Cooperative Agreement for development of the vaccine.

OBJECTIVE D: Develop a method for subtyping of *Campylobacter* strains.

PROGRESS D: We have previously reported on the use of sequencing a portion of the flagellin gene for typing of strains. This was based on the observation that a portion of the gene is highly variable. We have extended our research into the variability to discover that *Campylobacter* have a unique mechanism for sharing DNA sequence between two copies of the flagellin gene. The conclusion that can be drawn from these findings is that strains should have enough stability in the sequence so that epidemiologic studies can be performed without being confused by rapidly changing strain profiles. We have also embarked on studies to determine the genetic structure of *Campylobacter* populations. This will aid in the interpretation of subtyping, i.e., we

will be able to determine the origins of types and know if there is any special association of that type with certain diseases or sources.

IMPACT/TECH TRANSFER D: This research is being performed in collaboration with the Centers for Disease Control and Prevention, where plans are to use the sequence-based typing methods for human isolates. Standardization of *Campylobacter* typing is to be a subject of an upcoming international meeting and the sequence-based typing method will be presented as a promising technology.

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PREVENTION AND DETECTION IN LIVESTOCK OF POTENTIAL HUMAN FOODBORNE PATHOGENS (PCR-Based Techniques)

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OBJECTIVE A: To develop rapid and sensitive PCR-based techniques to detect and quantitate *Campylobacter*, *Listeria*, and other emerging human foodborne bacterial pathogens.

PROGRESS A: Members of the genus *Campylobacter* are major causes of human enteritis following consumption of contaminated food, water, or milk. Since there are few laboratory tests to differentiate these microbes, we have developed PCR-based rapid assays to aid in their identification. The thermotolerant *Campylobacter* include *C. jejuni*, *C. coli*, and *C. lari*. We have previously described a multiplex polymerase chain reaction to differentiate *C. jejuni* and *C. coli*. *Campylobacter lari*, like the other *Campylobacter* species, is found in water and in birds and has been implicated in human cases of bacteremia, diarrhea, and in at least one waterborne outbreak. We have now developed a PCR assay which targets the 16S rRNA genes for *C. lari*. The test is specific for *C. lari*. No PCR product is seen with strains of *C. jejuni*, *C. coli*, or other species of *Campylobacter*, *Arcobacter*, *Helicobacter*, *Escherichia*, *Salmonella*, or *Listeria*.

Campylobacter fetus causes bacteremia in immunocompromised individuals following consumption of contaminated dairy and raw meat products. Current methods to distinguish *C. fetus* from other *Campylobacter* species are limited and include growth at 25°C, resistance to nalidixic acid, and susceptibility to cephalothin. We have previously reported on DNA probes specific for *C. fetus* and have shown their specificity for differentiating isolates recovered from livestock. We have now developed a PCR-based probe sequence assay. The resultant PCR test only amplifies the 16S rRNA gene of *C. fetus*. It does not amplify bacterial species in the genera *Campylobacter*, *Arcobacter*, *Helicobacter*, *Escherichia*, *Listeria*, *Salmonella*, and *Wolinella*.

These PCR assays can yield reliable confirmation of *C. fetus* or *C. lari* within three hours after isolation of presumptive colonies on agar plates.

IMPACT/TECH TRANSFER A: These assays will contribute to the rapid identification of these fastidious pathogens and thus provide a better estimate of their role in causing human foodborne illness. These assays are simple to perform and thus are powerful adjuncts to conventional diagnostic testing.

OBJECTIVE B: To adapt rapid methods to detect *Salmonella* and other foodborne bacterial pathogens in mechanically separated turkey meat.

PROGRESS B: *Salmonella* rivals *Campylobacter* as a major cause of human foodborne illness. *Salmonella* causes human infection following consumption of contaminated foods, especially poultry products, including turkey. Current methods for isolation of *Salmonella* involve lengthy enrichments. We have adapted a PCR assay which amplifies all species of *Salmonella.* Using this assay, *Salmonella* species were detected in mechanically separated turkey meat (n=100 samples) throughout enrichment. *Salmonella* are identified on selective XLT4 agar by black colonies resulting from H_2S production. By using black colonies as an indicator of *Salmonella* contamination, we determined that 63% of the turkey samples were contaminated. When atypical colonies from non-H_2S producers were sampled by PCR, overall 80% of the samples were contaminated with *Salmonella*. We are now comparing our PCR assay with a commercially available PCR-based system with a shortened enrichment time.

Listeria monocytogenes is a foodborne pathogen with an estimated mortality rate in humans of 35%. Human outbreaks have been associated with consumption of contaminated poultry products. We have previously reported on a multiplex PCR to distinguish *L. monocytogenes* from other *Listeria* species. We adapted this multiplex PCR assay to identify *L. monocytogenes* in mechanically separated turkey meat samples (n=50). Samples were enriched in University of Vermont broths (UVM I, subcultured at 24 hours to UVM II) and subsequently streaked to Palcam agar. Suspect *Listeria* (colonies surrounded by black halos from aesculin hydrolysis) were picked and identified as *Listeria* species or *L. monocytogenes* by the multiplex PCR assay. *Listeria* species were isolated from 84% of the meat samples; *L. monocytogenes* from 56% of the samples. Studies are in progress to optimize the PCR assay to detect *Listeria* directly from enrichment. The sensitivity of our assay will be compared with that of a commercially available PCR assay with a shortened enrichment phase.

Enrichment of *Campylobacter* typically requires blood-based media, incubation in a low oxygen environment, and multiple incubation temperatures to maximize recovery of injured cells. We are evaluating two blood-free media, Rosef's and Tran's, for the recovery of *Campylobacter* from mechanically separated turkey meat samples. The PCR assay, which differentiates *C. jejuni* from other thermotolerant *Campylobacter* species (see Objective A), was used to detect *Campylobacter* in enrichments and from plate swipes. Pure cultures of *Campylobacter* seeded into each enrichment yielded distinct PCR products, thus indicating the suitability of PCR detection of *Campylobacter* directly from broths. The optimized assay was then used to screen for *C. jejuni* in mechanically separated turkey meat. Studies are in progress to remove the PCR inhibitors inherent in the high fat and protein content of meat enriched in both Rosef's and Tran's media.

Arcobacter spp. are aerotolerant *Campylobacter*-like organisms which have been recovered from livestock and meats, and have been associated with human enteritis. Of the four species of *Arcobacter, A. butzleri* is regarded as the human pathogen. *Arcobacter*, like *Campylobacter*, has been reported more frequently from poultry than from red meats, with recoveries ranging from

81% to 24% of poultry carcasses. We have previously described DNA probes to identify *Arcobacter* and *A. butzleri*. In this study we sampled turkey products obtained from three packing plants. *Arcobacter* was detected in 77% of mechanically separated turkey samples (n=395). Overall, *A. butzleri* was cultured from 56% of the samples examined. All isolates were confirmed by DNA probes. Differences in recovery rates were seen from samples obtained from plants A (77%), B (46%), and C (26%). This is analogous to the previously reported plant-to-plant variation which we observed in the recovery of *Arcobacter* spp. from ground pork. Genetic analysis of 121 isolates yielded 86 patterns, indicating multiple sources of contamination. It is of interest to note that despite its frequent recovery from poultry, we have been unable to experimentally infect 3-day-old chicks with *A. butzleri*. In contrast, age-matched birds were readily infected with *C. jejuni*. This indicates that multiple factors, including route of infection, infecting dose, age of the bird, etc., may be needed to establish *Arcobacter* infections in birds.

IMPACT/TECH TRANSFER B: These results indicate the suitability of PCR-based methods to identify potential human foodborne pathogens in livestock and in foods. The assays are highly reproducible and provide definitive identification in a shorter time than conventional biochemical testing. They are useful adjuncts in monitoring the effectiveness of HACCP programs.

OBJECTIVE C: To adapt PCR-based methods to the detection of potential foodborne pathogens in livestock.

PROGRESS C: Swine have been implicated as the principal reservoir of human pathogenic *Yersinia enterocolitica*, with the prevalence in pork tissues reported to be as high as 96.5%.

The purpose of this study was to utilize a PCR-based ELISA to estimate the prevalence of *Y. enterocolitica* in pigs obtained from three different sites in Iowa. The *ail* gene is a chromosomally-encoded virulence factor unique to *Y. enterocolitica* and was used to gauge pathogenicity. The PCR-ELISA bypasses the need for gel-based detection of PCR products. Fecal samples and swine tonsils were placed in three enrichments (PBS, Irgasin-ticarcillin-potassium chlorate, and modified trypticase soy broths) and subsequently plated to selective CIN agar. Colonies were picked and identified as *Y. enterocolitica* with PCR primers targeting the *ail* gene. *Y. enterocolitica* was detected in 83% of tonsil samples and in 43% of fecal samples obtained from farm I, but not in fecal or tonsillar samples from farm II or from swine raised in confinement at NADC. The PCR-ELISA will be evaluated with naturally contaminated ground pork samples. We are modifying a commercially available PCR system to shorten the interval from sample collection to final identification of *Y. enterocolitica*.

IMPACT/TECH TRANSFER C: Development of a rapid PCR-based assay will help define the prevalence of potential human pathogens in livestock and in meat. It will be useful in correlating the distribution of this pathogen with management practices.

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DIAGNOSIS AND EPIDEMIOLOGY OF BOVINE TUBERCULOSIS

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C.A. Bolin	CRIS Completion Date:	**September, 1997**

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OBJECTIVE A: Determine risk of infection with *Mycobacterium bovis* from ingestion of contaminated meat products.

PROGRESS A: We completed experiments using swine as an animal model to study the risk of humans becoming infected with *M. bovis* by ingestion of contaminated meat products. Swine have been used as an animal model to study a variety of human diseases. Results of our previous research demonstrated that swine are susceptible to *M. bovis* when challenged by the intratracheal, intratonsilar, intravenous, intragastric, and oral routes of inoculation. We determined that swine become infected when they are fed uncooked ground beef that contains between 1.2×10^2 and 5.7×10^7 *M. bovis* organisms. Infection was confirmed by histologic examination and bacteriologic culturing. Evidence of infection was observed in tissues collected from the head, thoracic and abdominal cavities, and carcass of swine challenged with *M. bovis* by the oral route.

IMPACT/TECH TRANSFER A: Results of this research demonstrate that a susceptible host can become infected with *M. bovis* by ingesting as few as 100 organisms in contaminated meat products that are not cooked .

PUBLICATIONS:

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DISEASE RELATED PROBLEMS OF POULTRY PRODUCTION AND PROCESSING (OSTEOMYELITIS IN TURKEYS, PROVENTRICULITIS IN BROILERS, AND INTESTINAL STRENGTH)

ARS Contact Persons:	CRIS Number:	**6226-32000-005**
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G.R. Huff	CRIS Completion Date:	**June, 2001**

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OBJECTIVE A: Determine the etiology of turkey green-liver/osteomyelitis complex (TOC), evaluate the involvement of the immune system and develop methods to reduce the impact of TOC on turkey production.

PROGRESS A: USDA-ARS research on TOC is focused on determining why a small percentage of healthy-appearing processed turkeys are infected with a variety of opportunistic bacteria, affecting bone, muscle, and synovial tissue. Previous studies conducted by this group suggest that immunological dysfunction contributes to the onset of TOC. We are developing a model for the reproduction of these lesions using intramuscular injection of dexamethasone and air sac inoculation with *E. coli* at 5 weeks of age. Dexamethasone is a synthetic corticosteroid which mimics the stress response and is known to suppress immune function. We hypothesize that differences in stress response of individual birds and resultant immunodeficiencies may be related susceptibility to these opportunistic infections. This is supported by the fact that TOC is only a problem in male turkeys, rarely in females, and the endocrine alterations involved in stress are influenced by gender. In an *E. coli* challenge study comparing male and female turkeys from the same hatch, stressed males had a higher incidence of infection and mortality than females.

IMPACT/TECH TRANSFER A: Our ability to experimentally reproduce the lesions of TOC is important for four reasons. First, field incidence of TOC is only 0.5%, a level too low for statistical evaluation of the effect of remedial measures such as antibiotic treatment or immunomodulation. This model should enable us to evaluate such measures. Second, this model is the first to demonstrate the possibility of a respiratory origin for the bacteria that cause TOC. Third, these data suggest that TOC might result from immune dysfunction and thus might be prevented by immunomodulation, and fourth, since *E. coli* air sacculitis/septicemia is considered by some to be the most important turkey disease, this model may have wide impact on turkey health. This research could lead to the reduction of TOC in turkeys, which would consequently reduce the need for the FSIS inspection procedures to identify affected carcasses.
We are currently investigating the efficacy of vitamin D treatment of young poults for the prevention of TOC. Supplemental vitamin D in the drinking water of stressed poults is currently used by the turkey industry to prevent bone weakness or field rickets. We are also currently

planning a cooperative study with industry to use our model to investigate the efficacy of certain antimicrobial agents known to enhance immune function.

OBJECTIVE B: To isolate and characterize the etiological agent of proventriculitis in broilers

PROGRESS B: Proventriculitis is a problem of food safety significance because rupture of the proventriculus during processing causes carcass contamination with intestinal contents. We have established that a filtrate of affected proventricular material which is free of bacteria will cause proventriculitis when fed to day-old broilers. We have also shown that copper sulfate added to feed can cause proventriculitis and can interact with the infectious homogenate to decrease body weights and feed conversion. We have succeeded in reproducing some of the lesions associated with proventriculitis with infectious bursal disease virus (IBDV) obtained by precipitating IBDV monoclonal antibody-virus complexes from our proventricular homogenate. We are currently investigating the involvement of another virus isolated in a cell-culture system.

IMPACT/TECH TRANSFER B: We are co-investigators on a patent application for development of a vaccine to prevent proventriculitis using IBDV isolated from proventriculus homogenates.

OBJECTIVE C: The development of methods to increase intestinal strength of poultry.

PROGRESS C: Intestines can become weakened and easy to tear due to the effects of disease, mycotoxins, and diet. Mechanical evisceration can then result in torn intestines which contaminate carcasses and increase the spread of potential pathogens throughout the processing plant. We have developed a sensitive method for accurately measuring intestinal strength not only to document the effects of agents which decrease intestinal strength, but also to evaluate ways to increase intestinal strength. Anecdotal field observations suggest that the treatment of feed with mold inhibitors (propionic acid) improves intestinal strength. In controlled studies we have been unable to demonstrate any benefit of propionic acid on intestinal strength. It was hoped that the effects astringents have on tissues would prove to be valuable to increase intestinal strength. However, neither tannic acid nor alum were found to have any effect on intestinal strength. Intestinal tissue is the most active tissue in animals with a very rapid cell turnover rate. It was believed that if the continuous irritation of these tissues, caused by the acids released for feed digestion, could be reduced with calcium carbonate, this would result in stronger intestines. Studies conducted using various levels of calcium carbonate in the feed have not demonstrated any beneficial effect on intestinal strength.

IMPACT/TECH TRANSFER C: The methodology developed for measuring intestinal strength is a valuable tool for research into intestinal disease. Development of methods to increase intestinal strength would decrease the cost of poultry processing and increase product safety.

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DEVELOPMENT OF MICROBIAL COMPETITIVE EXCLUSION METHODS TO REDUCE PATHOGENIC BACTERIA IN SWINE

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CRIS Number: **6202-42000-010**
FSIS Code: **C1AM04**
CRIS Completion Date: **October, 2001**

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OBJECTIVE A: Develop a competitive exclusion culture to prevent and/or control Salmonella in swine, and identify critical control points where competitive exclusion or other appropriate intervention strategies can be incorporated to control enteropathogens in commercial production of swine.

PROGRESS A: During the past year a new CRIS project was started aimed, in part, at using continuous-flow culture technology to develop a defined competitive exclusion culture for swine. Two different mirobial cultures using continuous-flow fermentation methods have been developed. Preliminary results indicate that both cultures significantly decrease the level of salmonellae colonization in the gut of baby pigs. The microbial composition of one of these cultures has been identified and contains fewer than 20 bacterial species. Studies are in progress to evaluate applications of these cultures at weaning, a potential critical control point.

IMPACT/TECH TRANSFER A: One of these competitive exclusion cultures has been approved by the agency for patent protection and an investigational new animal drug application (INADA) has been applied for through the Food and Drug Administration in order to perform field trials with a large integrated swine producer who has expressed interest in evaluating the efficacy of our competitive exclusion culture under commercial field conditions. A CRADA has been formed with an industry partner and provides for a postdoctoral scientist to ARS at the cost of the CRADA partner. Upon FDA approval of the INADA the continuous-culture fermentation will be scaled up and produced at a commercial GMP fermentation facility by our industry CRADA partners in order to supply product for commercial field trials. This research has been presented at several scientific meetings and journal publications are in submission.

OBJECTIVE B: Perform an epidemiology study on the presence of salmonellae and *Campylobacter* in commercial swine.

PROGRESS B: An agreement has been made and formal protocols approved for an epidemiology study to be performed in cooperation with The Texas Prison System (TPS). The TPS produces all of their own pork products and maintain approximately 5000 sows for

production. The TPS produce, growout, transport, slaughter and process 100% of the pork used in feeding the prison population within the TPS.

IMPACT/TECH TRANSFER B: The data obtained from this large study in cooperation with the TPS should allow us to have a more thorough understanding of the Critical Control Points for pathogen control in swine both pre and post harvest. Furthermore this data will allow us to evaluate the impact of intervention strategies such as competitive exclusion on the incidence and level of enteric pathogens in the animal, in the processing plant and in the final product. This study will be initiated in FY 98.

OBJECTIVE C: Determine the effect of antibiotics on the gut microflora of swine and how they impact the ability of the normal flora to protect the host animal from pathogen colonization, and the effects of antibiotics on development of antibiotic resistant pathogens in mixed microbial systems.

PROGRESS C: Models of the cecal flora of both antibiotic and non antibiotic fed swine have been developed using continuous-culture fermentation. Experiments examining the effect of antibiotics on the ability of the cecal microbial models to resist different salmonellae challenges have been initiated and preliminary results obtained suggest that the use of antimicrobials have a significant impact on the growth and survivability of salmonellae in mixed microbial gut colonization. Additionally, experiments have been initiated examining the survival and growth of antimicrobial resistant salmonellae within the mixed microbial models in the presence and absence of antimicrobials. Monoclonal antibodies specific to several different isolates within these mixed microbial models are in the developmental stage and experiments addressing specific interactions between normal flora and salmonellae will be initiated.

IMPACT/TECH TRANSFER C: By developing a better understanding of the impact of antimicrobials on normal gut microflora as well as enteric pathogens, recommendations and guidelines regarding types and levels of antimicrobials used in animal agriculture can be made. Additionally, these mixed microbial models should provide information on the effects of antimicrobials and other environmental influences on: 1) the ability of the host microflora to protect against colonization by enteropathogens and; 2) on populations of normal gut flora.

OBJECTIVE D: Develop a rumen model to predict the growth and survivability *of Escherichia coli* OI57:H7 in fasted and well fed cattle; develop a microbial based intervention strategy to inhibit the growth of *E. coli* O157:H7 in the rumen.

PROGRESS D: Continuous-cultures of rumen microflora have been developed from well fed and starved cattle. These cultures have been shown in our laboratory to be excellent *in vitro* models of *in vivo* rumen fermentation. Culture conditions have been created that models the growth, survivability and disappearance of *E. coli* within the rumen of starved and well fed cattle. A continuous-culture model of rumen microflora has been developed utilizing specific nutrients and environmental parameters that allows for the complete elimination of *E. coli* within

the culture. This information is being used to develop a microbial based intervention strategy aimed at suppressing the growth of *E. coli* O157:H7 in starved cattle prior to slaughter.

IMPACT/TECH TRANSFER D: Since cattle are routinely held off feed before sale and transport to slaughter, the effect of feed withdrawal and the microbiology of the rumen and of pathogen growth is critical. This research should help develop a cost effective microbial intervention strategy to use in pre-slaughter cattle that will suppress the growth of O157:H7.

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STRATEGIES TO CONTROL SWINE PARASITES AFFECTING FOOD SAFETY

ARS Contact Persons: **J. F. Urban, Jr., J. K. Lunney, D. S. Zarlenga**

CRIS Number: **1265-32000-049**
FSIS Code: **B2AA04,05**
CRIS Completion Date: **September, 2000**

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OBJECTIVE A: Reduce transmission of foodborne pathogens of swine by defining cytokine-regulated immune mechanisms that protect pigs against parasites that threaten food safety.

PROGRESS A: Control of *Toxoplasma gondii* infections in swine is a target for preharvest control of this foodborne pathogen. Efforts in this CRIS have focused on defining immune responses to *T. gondii* and other relevant parasitic infections, and to vaccination of swine with irradiated *T. gondii* oocysts. Swine infected with low numbers of *T. gondii* oocysts showed an early (day 6-8) increase in certain T cell populations as well as increases in specific cytokine levels, specifically IFN-gamma. Pigs vaccinated with irradiated oocysts were protected against both high and low dose *T. gondii* oocyst challenge. However, these pigs were not fully protected because some cyst development was detected; antibody levels were not predictive of the success of vaccination.

Basic immunological studies in rodent models have demonstrated the prophylactic and therapeutic effects of recombinant cytokines in the control of parasitic infection. The interaction of cell surface molecules on T cells and macrophages (CD28/CTLA4/CD80/CD86) are predictors of successful responses to parasitic infections. Swine infected with whipworm have greater susceptibility to microbial infection including *Campylobacter* spp. that threaten human health.

IMPACT/TECH TRANSFER A: These studies should help to determine whether specific cell subsets or cytokines are potential predictors of positive *T. gondii* vaccination. More effective vaccination protocols against *T. gondii* infection will require information on methods of boosting swine immunity to infection.

Assays for detecting swine cytokine responses and cell subset changes during infection will help scientists enhance immunity to a variety of infectious diseases.

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PREVENTION & THERAPY FOR PROTOZOAN PARASITES AFFECTING FOOD ANIMALS, FOOD SAFETY, PUBLIC HEALTH

ARS Contact Persons:	CRIS Number:	**1265-32000-050**
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OBJECTIVE A: Prevent illness in food animals, control food contamination and protect public health from protozoan parasites. *Cryptosporidium parvum* affecting all mammals will be a target of immunity based strategies.

PROGRESS A: A series of experiments was conducted that demonstrated eastern oysters from the Chesapeake Bay will remove *Cryptosporidium parvum* oocysts from artificial seawater and store them in an infective state in gills, hemocytes and gut for up to 1 month.

Our previously developed polymerase chain reaction (PCR) technique has been used as a rapid and sensitive method for measuring infection levels of cryptosporidial stages in intestines of neonatal mice.

We assessed the cytokine immune response in intraepithelial, lamina propria cells and draining lymph nodes of 9-day old calves infected with *Cryptosporidium parvum*. The infection elevated the interleukin-12 and gamma interferon intraepithelial cells, indicating that these cytokines could be stimulated early in life, and that infection could possibly be prevented in young calves.

Oocysts of *C. parvum* were irradiated at 5 unit increments from 10 to 20 Krads and tested for infectivity in neonatal mice. It was found that 20 Krads rendered oocysts noninfectious.

IMPACT/TECH TRANSFER A: If oysters, under experimental conditions, can filter oocysts from seawater and hold them internally for a month while they remain infectious, it is possible that oysters can be vectors for *Cryptosporidium parvum* by becoming infected under natural conditions and this possibility should be explored for public health reasons. The PCR test has application for diagnosis purposes and could be shared or licensed to interested parties. The finding that IL-12 production is stimulated in neonatal calves, taken with our earlier findings in mice that IL-12 protects against cryptosporidiosis, could lead to development of a prophylactic treatment for calves. The finding that 20 Krads of radiation will kill oocysts can be applied to sterilization of potentially contaminated food products.

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IDENTIFICATION AND MAPPING OF GENES INVOLVED IN PARASITIC DISEASE RESISTANCE/SUSCEPTIBILITY

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FSIS Code: **B2AA05**
CRIS Completion Date: **September, 2000**

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OBJECTIVE A: Identify and breed livestock and poultry that are genetically resistant to parasite infections.

PROGRESS A: Outbred swine and genetically defined minipigs, pigs with known swine leukocyte antigen haplotypes, were tested to determine whether *Toxoplasma gondii* resistant pigs could be identified. Several inbred pigs which were resistant were identified. Tissue samples from each group of pigs were saved so that immune mechanisms controlling this resistance could be defined using assays for cytokine expression. Preliminary tests indicate that interferon-γ is expressed but comparative analyses need to be performed to determine whether this cytokine controls parasite resistance. Such assays are underway. Once data has been collected on enough individuals, mapping will be pursued to determine what other genes and immune factors encode such resistance.

IMPACT/TECH TRANSFER A: These studies should help breeders to reduce costs of drug and vaccine treatments by selecting for parasite resistant stock. In areas where these parasitic diseases cannot be eliminated, this alternate approach should result in healthier pigs and should help prevent parasite contamination of pork products.

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EPIDEMIOLOGY AND CONTROL OF *TOXOPLASMA*, *TRICHINELLA* AND RELATED PARASITES IN DOMESTIC ANIMALS

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CRIS Completion Date: **March, 1999**

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OBJECTIVE A: To assess the feasibility of a pre-harvest certification program for trichinae infection in pigs.

PROGRESS A: Studies in New England and New Jersey were completed during FY97 and the results of these studies summarized and submitted for publication. The enzyme immunoassay was shown to be highly sensitive in detecting low level infections in pigs. The prevalence rates found by serology were 0.47% in New England states and 0.26% in New Jersey. These rates are about one-half those found ten years previously using a less sensitive test. Worms were recovered from some of the seropositive pigs; worm infection levels were always < 1 larvae per gram of tissue and thus were not sufficient to pose a public health risk. Risk factors for infection on these farms were found to include exposure to rodents, wildlife and wildlife carcasses. A certification pilot was initiated at SiouxPreme Packing Company in Sioux Center, Iowa in April, 1997. The pilot has two phases: **I.** Testing approximately 250,000 hogs for trichinellosis using pooled sample digestion and ELISA methods; and **II.** Conducting risk factor audits on approximately 250 farms which have sold tested pigs to SiouxPreme during the course of the study. The farm audit was developed in cooperation with APHIS, Center for Epidemiology and Animal Health. Veterinary practitioners from Iowa, South Dakota and Minnesota were trained to administer this audit. Testing of hogs will be completed in October, 1997 and farm audits will be completed about the same time. Analyses of farm audits will be used to determine the cut-off of scores necessary to rate a farm as free of risks for trichinae transmission. Certified and non-certified farms will be compared with testing results.

IMPACT/TECH TRANSFER A: The results of the initial portion of the study supported the use of ELISA for identifying infected pigs and provided a basis for assignment of risk factors for transmission of trichinellosis. Completion of the Iowa study will provide a framework for further development of a certification program for the pre-harvest control of an important foodborne pathogen in swine and will serve as a model for programs for other zoonotic diseases.

OBJECTIVE B: Provide training and quality control in a program for the inspection of pork and horsemeat for export to the European Union and Russia; to provide program support in the form of evaluating and improving inspection methods for trichinae in pork and horsemeat.

PROGRESS B: The ARS administers a training and quality control program which includes 7

pork packers and 5 horse packers which ship certified trichinae-free pork and horse meat to Europe and Russia. Three to four training sessions are held each year for personnel at participating plants to become certified. On a quarterly basis, check samples are prepared and distributed to all certified trichinae analysts. Accurate analysis of check samples allows continued certification of these inspectors. Research projects to determine the effectiveness of digestion and serology methods for the detection of trichinellosis, primarily in pigs, continue to be performed. This research is used in making modifications to the existing program and is conducted in cooperation with scientists in Canada, Europe and Russia. In FY97, experiments were conducted to determine the feasibility of using blood or tissue fluids in the ELISA in place of serum (due to difficulties in obtaining serum samples at slaughter plants). Either blood or serum, at adjusted concentrations, were found to perform as well as serum in the ELISA. Further studies are being conducted on the use of fresh and frozen tissue for recovering fluids suitable for testing in the ELISA. The use of tissue fluids is consistent with existing technology for conducting an ELISA for *Salmonella*.

IMPACT/TECH TRANSFER B: The results of this program and related research is the assurance of market access for fresh pork and horse meat products in Europe and Russia.

OBJECTIVE C: To assess the national prevalence of trichinellosis in hogs in the United States.

PROGRESS C: Using sera collected from the 1990 and 1995 National Animal Health Monitoring Survey (NAHMS) for swine, we tested for antibodies to *Trichinella spiralis* by ELISA. The prevalence of trichinellosis based on these results was 0.098% in 1990 and 0.013% for 1995. These prevalence rates reflect declines in the national prevalence of trichinellosis in swine, a fact which is further reinforced by the use of a test which was much more sensitive than those used previously. Analysis of NAHMS survey results demonstrated that farms which had seropositive animals had management practices which included risk factors for transmission of trichinae. Further efforts to develop an even more accurate national prevalence for trichinellosis will require a larger sample size collected from slaughter plants.

IMPACT/TECH TRANSFER C: The availability of national prevalence data will provide the pork industry with an estimate of the magnitude of the problem to be addressed and assist in making decisions concerning control programs.

OBJECTIVE D: Develop a low-cost assay to detect antibodies to *Toxoplasma*.

PROGRESS D: Recombinant *Toxoplasma gondii* antigens B427, V22, C55, and C51 were assessed for their potential for use in ELISAs for diagnosis of toxoplasmosis in swine. The antigens were evaluated with sera from young pigs which had been fed 1 to 10,000 *T. gondii* oocysts. Results were compared with enzyme linked immunosorbent assays (ELISA) based on a native *T. gondii* antigen extract. All 4 recombinant antigens produced strong responses with sera drawn at 4 wk post-inoculation (PI). However, there was significant animal to animal variation in responses to the individual antigens. Enzyme linked immunosorbent assays based on a combination of the four antigens produced consistently elevated responses in all experimentally

infected pigs. These levels could be detected from 4 through 42 wk PI. In comparison to ELISAs based on the native antigen extract, ELISAs based on a pool of all 4 recombinant antigens produced higher readings at 4 wk PI and retained these levels throughout the test period in both low and high dose inoculum pigs, but had somewhat greater pig to pig variation.

IMPACT/TECH TRANSFER D: Efforts are underway to test more recombinant proteins of *T. gondii* in order to raise the sensitivity of the test. This test will be useful for evaluating the *Toxoplasma* status of pigs for certification purposes.

OBJECTIVE E: Determine control procedures for toxoplasmosis in swine.

PROGRESS E: Pigs infected with *Toxoplasma gondii* are considered an important meat source of infection for humans in the U.S. At present there is no vaccine to prevent *T. gondii* infection in pigs. Recently we found that *T. gondii* oocysts inactivated by gamma irradiation retained immunogenicity for mice. Mice fed oocysts irradiated at 0.20 or 0.40 kGy of ^{137}Cs were protected against a lethal oral challenge with *T. gondii* oocysts. Therefore, the possibility of immunization of pigs with irradiated *T. gondii* oocysts was investigated. In 2 experiments 23 vaccinated 2-3-mo-old pigs were fed (11 pigs only once and 12 pigs twice) ~10^5 oocysts irradiated at 0.3 or 0.4 kGy of ^{137}Cs and 11 pigs served as unvaccinated controls. Eleven to thirteen weeks later 15 vaccinated and 5 non-vaccinated pigs were challenged orally with a high dose (10^5 or 10^6) of oocysts, and 7 vaccinated and 6 non-vaccinated pigs were challenged orally with a low dose (10^2 or 10^3); 1 vaccinated pig was not challenged with oocysts. All non-vaccinated pigs challenged with high dose became ill starting 4-5 days post challenge (p.c.) and 1 died day 9 p.c., 1 died day 16 p.c. and 1 was killed day 10 p.c. because of weakness, whereas all vaccinated pigs and non-vaccinated pigs challenged with low dose, and the 1 vaccinated unchallenged pig remained clinically normal. Bioassay of pig tissues (tongue, diaphragm, brain) in mice indicated fewer tissue cysts in tissues of vaccinated pigs compared with unvaccinated pigs following challenge with live oocysts. However, vaccination with 1 or 2 doses of irradiated oocysts did not completely prevent formation of tissue cysts even in pigs challenged with 10^2 oocysts.

IMPACT/TECH TRANSFER E: The results of this initial study in pigs suggests that it may be possible to develop a vaccine to reduce *T. gondii* infection in pigs which will aid the pork industry in efforts to produce pathogen free pork.

OBJECTIVE F: Preliminary studies on the survival of *Toxoplasma* tissue cysts in salt solutions.

PROGRESS F: The effect of storage of *Toxoplasma gondii* tissue cysts in various NaCl solutions at different temperatures was studied. Tissue cysts from rodent brains were suspended in 0.85%, 2.0%, 3.3%, and 6.0% aqueous NaCl solutions. After storage at 4 to 20° C for various time intervals, brains were bioassayed in mice for viability of *T. gondii*. At 4° C, tissue cysts survived for at least 56 days in 0.85% NaCl, for 49 days in 2.0% NaCl, and for 21 days in 3.3% NaCl solutions. At 10° C, tissue cysts survived for at least 21 days in 0.85%, 2.0%, and 3.3% NaCl solutions. At 15° C, tissue cysts survived for at least 21 days in 0.85% NaCl, 14 days in

2.0%, and 3.3% NaCl solutions. At 20° C, tissue cysts survived for 14 days in 0.85% NaCl, 7 days in 2.0% NaCl, and 3 days in 3.3% NaCl solutions. Tissue cysts generally did not survive in 6.0% NaCl solution at any temperature. Based on USDA regulations, requiring a minimum application of 5% salt at various time/temperature combinations, *T. gondii* would be inactivated as determined by the data presented here.

IMPACT/TECH TRANSFER F: This information will be useful to FSIS in setting standards for the processing of meat products to inactivate *T. gondii*.

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CONTROL AND PREVENTION OF *CRYPTOSPORIDIUM PARVUM* INFECTION

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CRIS Number: **3625-32000-006**
FSIS Code: **N2AM01**
CRIS Completion Date: **February, 1999**

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OBJECTIVE A: Develop methods to prevent or minimize *Cryptosporidium parvum* infection in cattle.

PROGRESS A: We tested the efficacy of an experimental vaccine for cryptosporidiosis in calves in a commercial dairy. We found that the intense, early exposure of calves to the parasite in a highly contaminated farm environment overcame any potential protection induced by the vaccine. We are now working to develop a second generation vaccine that will provide quicker protection and thus be more effective in a production setting.

IMPACT/TECH TRANSFER A: These findings indicate that protection of calves from on-farm contamination with *C. parvum* will require better vaccines and treatments along with improved hygiene and management of calves by producers to reduce early exposure to the parasite. To this end, we have written articles and presented seminars aimed at producers and veterinarians outlining ways to reduce the impact of *C. parvum* infection through management strategies. These efforts will reduce the levels of preharvest contamination, and reduce the chance of *C. parvum*-contaminated water and food reaching the consumer.

OBJECTIVE B: Develop methods to control contamination of the environment by animals infected with *C. parvum*.

PROGRESS B: We are examining the sources of *C. parvum* in the environment of the newborn calf. We found that on a highly contaminated dairy, the parasite could not be found in the adult cows, nor in the soil of barns and pens. However, parasites were found in scrapings from the walls of wooden pens where *C. parvum*-infected calves were held.

IMPACT/TECH TRANSFER B: These findings indicate that moist, porous surfaces are a likely source of new infections for calves. Thus, we have recommended to producer groups and veterinarians that stalls be cleaned and thoroughly dried after housing infected calves before being used to house newborn, noninfected calves. These efforts will reduce the early exposure of calves to the parasite, thus breaking the cycle of transmission, and further environmental contamination.

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Part II. PATHOGEN CONTROL DURING SLAUGHTER AND PROCESSING (Inspection Technology)

CONTROL OF PATHOGENIC AND SPOILAGE BACTERIA ON RED MEAT (Carcass Washing Systems)

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CRIS Number: **5438-32000-014**
FSIS Code: **M2MI01**
CRIS Completion Date: **April, 2001**

OBJECTIVE A: Incorporate the GRAS status bacteriocin, nisin, into plastic films for preservative antimicrobial efficacy.

PROGRESS A: Nisin was successfully incorporated into the formulation of polyethylene-based plastic. This first batch of nisin-impregnated plastic (NIP) was produced with 1.0 mg of nisin per ml of formulation. The plastic film was determined to have retained antimicrobial activity as determined by agar plate inhibition assays.

Preliminary experiments using a second batch of nisin-impregnated plastic (NIP) as a wrap for inoculated meat samples were conducted. This batch was produced with 0.5 mg of nisin per ml of formulation. In these trials UV-sterilized meat samples inoculated with the spoilage bacterium *Brochothrix thermosphacta* were wrapped in the NIP and then vacuum packaged. Samples were stored at 4°C for up to 20 days. Half of the samples were subjected to simulated temperature abuse by being shifted from 4°C to 12°C after 2 days of 4°C storage. These were compared to inoculated meat samples that were not wrapped in NIP but were vacuum packaged.

Results indicated the NIP effected an initial 2.5 log cfu/cm^2 reduction in initial microbial load of the inoculated meat. This reduction carried through the remainder of the 20-day storage period. By day 20 the 4°C NIP wrapped samples had a *B. thermosphacta* count of log 6.0 cfu/cm^2 vs the control log 7.0 cfu/cm^2. Temperature-abused samples had similar counts (NIP samples were 90% or log 1 lower than the control samples), but they reached these final levels approximately eight days before the strict 4°C-held samples.

These results indicate that nisin does not lose its antimicrobial activity when incorporated into plastic films. Data suggest that the antimicrobial effects and shelf-life or pathogen reduction

efficacy of the NIP might be the result of an immediate reduction in the microbial count which is carried through the storage period. Work will proceed to utilize this technology for vacuum packaging refrigerated meat subprimals.

IMPACT/TECH TRANSFER A: To our knowledge this is the first demonstration that an antimicrobial peptide (food grade) can be incorporated into a synthetic petroleum-based plastic. While much research remains to be done regarding the plastics chemistry and bacteriocin activity retention in plastic, the two obvious avenues are to utilize other bacteriocins with a wider range of antimicrobial activities and to utilize corn- or soy-based plastics as the carrier film.

OBJECTIVE B: Determine the long term effect of alkaline, organic acid, or hot water washes on the microbial profile of refrigerated beef contaminated with bacterial pathogens post-wash.

PROGRESS B: The effect of 2% (vol/vol) lactic acid (LA), 2% (vol/vol) acetic acid (AA), 12% (wt/vol) trisodium phosphate (TSP), 72°C water (HW), and 32°C water (W) washes on bacteria populations introduced to beef carcass surfaces post-treatment was determined up to 21 d at 4°C vacuum packaged storage. Beef carcass short plates were collected from cattle immediately after harvest and subjected to the above treatments or untreated (C). Short plates were then inoculated with low levels (ca.<2 $\log_{10}$) of *Listeria innocua*, *Salmonella typhimurium*, *E. coli* O157:H7, and *Clostridium sporogenes* contained in a bovine fecal cocktail. In general, growth of these four bacteria, aerobic bacteria, lactic acid bacteria, and pseudomonads was suppressed or not observed when LA or AA treatments were used. Bacteria introduced to treated beef tissue after the tissue had received a TSP treatment demonstrated some growth suppression, but to a lesser extent than on acid-treated tissue, and in some cases grew as well as they did on untreated beef surfaces. Following an immediate reduction, hot water or water washes offered little growth suppression of pathogens during subsequent storage when these bacteria were introduced to beef tissue post-treatment. The use of a final lactic or acetic acid wash during the processing of beef carcasses offers some residual efficacy in suppressing pathogen proliferation during refrigerated storage, should these bacteria be introduced immediately after carcass processing.

IMPACT/TECH TRANSFER B: The incorporation of various antimicrobials as total carcass treatments is presently in limited use by the beef processing industry. It is important that the effectiveness of these processes be determined during long term storage of primals that have received post-process contamination. The industry will be able to use this information to determine the best intervention process to incorporate into a HACCP-based process for their product line. This information has been disseminated via presentations made to professional and industrial groups and has been submitted for publication in a professional journal.

OBJECTIVE C: Determine the long-term bacterial profile of refrigerated ground beef made from carcass tissue, surface contaminated with pathogens before receiving hot water, alkaline, or organic acid washes.

PROGRESS C: Beef carcass surface tissue was inoculated with bovine feces containing one of two different levels (ca. 4 or 6 log cfu/ml) of *Escherichia coli* O157:H7, *Listeria innocua*, *Salmonella typhimurium*, and *Clostridium sporogenes*. The tissue was then subjected to one of several possible carcass wash intervention treatments. The treatments were 2% (vol/vol) DL-lactic acid (LA), 2% (vol/vol) acetic acid (AA), 12% (wt/vol) trisodium phosphate (TSP), 74± 2°C at the tissue surface hot water (HW), or 32± 2°C at the tissue surface water (WW). A control group was left untreated. After treatments, beef tissue was cold-stored for 24 h, then ground and incubated at 4 or 12°C for up to 21 or 3 d, respectively. When samples were held at 12°C for 3 d, AA treated samples maintained a significantly lower aerobic plate count than the control and the lowest levels of pseudomonads of all samples tested. After being held at 4 or 12°C for 21 or 3 d, respectively, antimicrobial treated samples had lower levels of *E. coli* O157:H7, *L. innocua*, *S. typhimurium*, and *C. sporogenes* than beef treated with a WW or the untreated control. In several cases samples exhibited no detectable presence of any of these bacteria. Ground beef produced from tissue treated with HW yielded lower populations of these bacteria when compared to WW or untreated control, but the populations were generally higher than those observed in any of the antimicrobial treated samples. These trends continued throughout all storage conditions over time. Results from this study indicate use of carcass interventions presently available to the slaughter industry can be helpful in producing safer ground beef. In particular, the residual effects of chemical antimicrobials will offer the best long-term protection.

IMPACT/TECH TRANSFER C: The incorporation of various antimicrobials as total carcass treatments is presently in limited use by the beef processing industry. It is important that the effectiveness of these processes be determined during long-term storage of the resulting ground beef from carcasses that have received post-process contamination. The industry will be able to use this information when determining the best intervention process to incorporate into a HACCP-based process for their product line. This information will be disseminated via presentations made to professional and industrial groups and a publication in a professional journal.

OBJECTIVE D: Determine the effect of rapid desiccation with heat at one or two points in the slaughter process for reducing bacteria on beef carcass surfaces.

PROGRESS D: A series of experiments was conducted to determine the effectiveness of rapid desiccation with heat at one or two points in the slaughter process to reduce bacterial contamination and/or attachment on beef carcass surfaces. In the first experiment, beef surfaces were inoculated with bovine feces and water washed (A; 125 psi, 15 s, 35°C); desiccated (400°C, 15 s) before inoculation and subjected to a water wash (B); inoculated, water washed and desiccated for 30 s (C); or desiccated, inoculated, water washed, and desiccated for 30 s (D). Remaining bacterial populations of samples treated with D exhibited the fewest populations of APC, coliforms, and *Escherichia coli*. When *E. coli* O157:H7, *Salmonella typhimurium, Listeria innocua,* and *Clostridium sporogenes* were monitored following treatments with D, none of the organisms was detected. An additional set of experiments was conducted with less heat (300°C)

for shorter times to minimize surface discoloration. When desiccation (300°C) was conducted for 10, 12, or 15 s prior to fecal contamination and followed by a water wash, it was demonstrated that none of the treatments was significantly different from the others for reducing APC from shortplates; however, the 10 s treatment was preferred for its shorter time. When desiccation for 10 s was combined with water washing and followed by a second desiccation step (300°C) for 15, 20, or 25 s, populations of APC, coliforms, and *E. coli* were reduced to the greatest extent when the second desiccation step was applied for 25 s. In all cases, the desiccation step(s) and water wash combinations were more effective than water washing alone for reducing bacterial contamination on beef surfaces.

IMPACT/TECH TRANSFER D: Based on the results of this study, the initial desiccation procedure affects bacterial attachment to beef surfaces and the combination of desiccation before inoculation and immediately after spray wash treatments was demonstrated to be more effective than water washing alone for reducing undesirable bacteria on beef surfaces.

OBJECTIVE E: Determine whether a commercially available meat binding system (Fibrimex®) is a more effective carrier or delivery system for the GRAS status bacteriocin nisin to meat surfaces than direct application.

PROGRESS E: Lean beef carcass tissues were inoculated with the meat spoilage bacterium, *Brochothrix thermosphacta* (Bth.), then left untreated (U), treated with 10 μg/ml nisin (N), Fibrimex® (F), or 10 μg/ml nisin immobilized in Fibrimex® (FN). Treated tissues were held at 4°C and populations of *B. thermosphacta* and nisin activity were determined at 0, 1, 2, and 7 days.

Nisin in Fibrimex® reduced the levels of Bth. to undetectable levels (0 $\log_{10}$ cfu/cm^2) at day 0 and throughout the study, while populations of U-, F- and N- treated tissues exhibited increased bacterial growth over time.

IMPACT/TECH TRANSFER E: This study demonstrates that delivery of a bacteriocin on the surface of beef with an edible meat binding agent was a more effective delivery system for a bacteriocin than direct application. Fibrimex® is currently used to produce structured meat products. These products result in surface areas of meat actually being internalized when bound together with the binding agent. An effective antimicrobial compound in the binding agent could enhance product shelflife and microbial safety.

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CONTROL OF PATHOGENIC AND SPOILAGE BACTERIA ON RED MEAT
(Parameters for Reconditioning Accidentally Contaminated Meat and Poultry)

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CRIS Number: **5438-32000-014**
FSIS Code: **M2MI01**
CRIS completion Date: **April, 2001**

OBJECTIVE A: Develop rapid means to concentrate bacteria from beef carcass and ground beef samples to enhance pathogen detection limits.

PROGRESS A: Non-cultural enrichment methods were utilized to rapidly remove bacteria from suspension that did not rely on centrifugation and could potentially be used with large sample volumes. Attempts to concentrate bacteria using hydrophobic chromatography media matrixes and perfluorocarbon emulsion droplets with attached lectins both failed.

Experimentation revealed that hydroxyapatite crystals (HA) had a very high binding affinity for a number of bacterial pathogens including *Salmonella* spp., *E. coli, Listeria* spp., *Staphylococcus aureus*, and *Yersinia enterocolitica*. Pathogenic bacteria in suspension, bacteria from beef carcass sponge samples and bacteria from bovine fecal samples were bound to HA at levels as high as 99% of the original population.

Current efforts are centered on incorporating the hydroxyapatite concentration method with a PCR (polymerase chain reaction) assay for *Salmonella* spp. Two questions remain to be answered: will the HA method decrease the time of assay detection by presenting the minimum threshold level of cells for the assay, and will HA concentration function in a PCR-based method used on actual carcass spongings and ground beef.

IMPACT/TECH TRANSFER A: The potential impact of this procedure is a means to shorten assay time on samples that normally require several hours of growth-based enrichment. This would include carcass surface samples and ground beef, but could potentially be used for other species including poultry rinses and pork and lamb. Although we are incorporating the HA procedure with a PCR test, other test formats could benefit by this protocol including ELISA and culture-based methodology.

OBJECTIVE B: Determine the relationship between the aerobic plate count class of a beef carcass and the likelihood of isolating generic *E. coli* from that sample for use as a rapid HACCP monitor of the dressing procedure.

PROGRESS B: Research to date is based on a total of 535 sponge samples collected from the past three years from three different beef plants (one fed beef and two cow/bull processing plants). Aerobic plate counts were classified into 4 groups; 0-100; 101-1000; 1001-10,000; and > 10,000 aerobic bacteria per cm^2 represented as classes 1, 2, 3, and 4, respectively.

Overall, class 1 samples had a 15% isolation rate of generic *E. coli* while classes 2, 3, and 4 had rates of 42%, 73% and 96%, respectively. Chi-square association univariate analysis indicated a strong relationship ($x^2 = 74.08$, $p<0.001$) between APC class and incidence of *E. coli* isolation from that sample.

Also determined by univariate analysis (x^2) were strong associations between APC class and incidence of *E. coli* isolation from samples sorted by in-plant location. Post-wash (pre-chill) and chilled carcass samples had a significant degree of association ($x^2 = 26.13$ and 9.01, $p<0.001$, respectively, for chilled and post wash samples) while pre-wash samples had a less clear association ($x^2 = 1.45$, $p=0.69$).

At present there appears to be no need for further sampling. Multiple logistic regression analysis of the dataset indicated an influence of the location (pre-wash, post-wash or chilled) as well as the source (plant 1, 2, or 3). Accounting for these covariates (source and location) in a multivariate model supported the hypothesis that the greater the APC class, the more likely the potential for a beef carcass surface sample to harbor generic *E. coli*. Testing the multivariate model using the continuous variable of the log APC/cm^2 also supported the hypothesis that the greater the level of APC, the more likely the potential for a beef carcass surface sample to harbor generic *E. coli*.

IMPACT/TECH TRANSFER B: This data analysis supports the assertion that assessing the level of APC on a carcass is an indicator of the likelihood of also finding *E. coli* in that sample. Utilizing means to rapidly gauge carcass contamination levels could provide the processor with a HACCP monitor with which process breakdowns or deviations leading to fecal contamination would be detected within minutes of first happening.

OBJECTIVE C: Develop a simple and representative purge sampling method to detect total aerobic bacteria and *Escherichia coli* O157:H7 in raw beef combos.

PROGRESS C: The purge from beef combos (a boxed collection of beef trimmings) was tested as a means of representatively sampling the microbial content of this raw product. In the first experiment, purge was sampled from model beef combos that had been inoculated with bovine feces. Data from this experiment indicated a linear correlation ($r = 0.94$) between the total aerobic bacteria counts derived from the purge samples of a model beef combo and the total aerobic bacteria present in a rinse sample of the entire model beef combo. In a second

experiment, two 500-g meat pieces were inoculated with an antibiotic-resistant *Escherichia coli* O157:H7 and placed at various levels within a 75-cm meat column. The marked bacteria were retrievable from the purge of the meat column after 24 h, demonstrating that bacteria are carried downward into the purge. During the third part of the study, 90 beef combos (ca. 900 kg beef/combo) were randomly selected at the receiving dock of a commercial grinding facility and sampled using both purge and concurrently used 11-g core samples. Purge samples from these combos recovered significantly greater numbers of mesophilic and psychrotrophic aerobic bacteria, coliforms, and *E. coli* than core samples from the same combos. Additionally, coliforms and *E. coli* were recoverable from 100 and 80 percent, respectively, of the purge samples taken, while core samples were only able to recover 60 and 40 percent, respectively, from the same combos. These findings indicate that a purge sample from a beef combo is a more efficacious sampling method for determining the general bacterial profile and identifying the presence of coliforms and *E. coli* than randomly taken core samples.

IMPACT/TECH TRANSFER C: The successful validation of this method has impact on the production of ground beef. This information has been disseminated via presentations made to professional and industrial groups and has been accepted for publication. Interest in implementing this sampling technique has been expressed by several industry people.

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REDUCTION OF BACTERIAL CONTAMINATION AND PATHOGEN LOAD DURING POULTRY PROCESSING

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CRIS Number: **6612-41420-004**
FSIS Code: **R3MC06**
CRIS Completion Date: **August, 1999**

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OBJECTIVE A: Study the formation and composition of biofilms on processing plant surfaces.

PROGRESS A: Studies to describe the formation of biofilms and the importance of pathogens within the biofilms in the processing plant environment were continued. Methods to measure attached bacteria from broiler carcass rinse to poultry processing equipment surfaces were improved. Rubber picker finger material was found to be the least conducive surface to bacterial attachment from an array of surface materials from the poultry processing plant. Digital aroma technology was used to detect bacterial contamination and classify microorganisms from the poultry processing plant. Bacterial isolates important to poultry processing as potential pathogens were isolated and identified.

IMPACT/TECH TRANSFER A: Increasing the use of materials that do not support growth and attachment of microorganisms will improve food safety. Identifying the factors that play a role in pathogen attachment is a necessary step toward determining the relative importance to food safety of pathogens found in the poultry processing plant.

OBJECTIVE B: Develop methods to prevent the formation of or facilitate removal of biofilms on processing plant surfaces to allow efficacious cleaning and sanitizing.

PROGRESS B: Evaluation of protocols for testing the resistance of a mixture of organisms found in the whole carcass rinse to a range of disinfectants and sanitizers commonly used in the food industry continued. Equipment for on-line processing of visually contaminated carcasses was tested and reduced the number of carcasses being subjected to off-line reprocessing. There were no significant effects from on-line treatment for aerobic plate counts, *Salmonella*, *Campylobacter* or coliforms.

IMPACT/TECH TRANSFER B: Inhibiting bacterial attachment will enhance food safety by preventing the increase in bacterial numbers necessary for biofilm formation. Finding the least amount of treatment necessary to effectively inhibit biofilms will be economical for the industry and consumers as well as reduce the impact of agriculture on the environment.

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ENGINEERING INNOVATIONS AND MICRO DEVELOPMENTS TO REDUCE CONTAMINATION OF POULTRY AND EQUIPMENT

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CRIS Number: **6612-41420-003**
FSIS Code: **R3IM01-05**
CRIS Completion Date: **October, 1999**

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OBJECTIVE A: Develop methods to remove feathers post-mortem from poultry carcasses.

PROGRESS A: Manuscripts describing affects of ante-mortem orientation and stunning methods on feather retention force (FRF) have been accepted and are in press. In summary, only minor changes in FRF could be detected ante- and peri-mortem, and no significant factors influenced post-mortem FRF. Plans for cutaneous nerve denervation are scheduled this fall with cooler weather for housing birds post operative. The spray scalder was tested in conjunction with electrical and carbon dioxide stunning for defecatory response and microbial contamination. Differences were noticed between stunning methods in defecatory response and the amount of adhering fecal matter but with slightly lower picking scores. In one replication, broilers stunned with carbon dioxide and spray scalded demonstrated significantly lower carcass microbial recovery for total coliforms ($\triangle$0.77 logs) and total *E. coli* ($\triangle$0.7 logs). Spray scalder modifications are being made to increase coverage without increasing water usage.

IMPACT/TECH TRANSFER A: Results demonstrated that stunning methods, electrical or carbon dioxide, did not influence post-mortem FRF or efficiency of carcass defeathering. Immersion and spray scalding generally resulted in comparable carcass defeathering scores and microbial recovery. Successful completion of the spray scalder design with comparable picking efficiency, would be used by the poultry industry for reduced water usage as well as significantly less cross-contamination than with the communal bath scalders now in use.

Electrical stimulation of poultry during bleed out has been introduced into more than 12 commercial processors in the U.S. and one in Brazil using design work done by our reasearch unit. Electrical stimulation has a two fold advantage in that the rate of rigor mortis is accelerated thus allowing the carcasses to be deboned earlier and handled less. By decreasing the holding time for carcasses prior to packaging there is less time for microbial growth and decreased handling that may cause further contamination or cross contamination. The second advantage is that over 87% of stimulated carcasses defecate during stimulation. This defecation voids the lower gut and reduces the possibility of carcasses being contaminated with fecal matter and associated enteric pathogens during evisceration and other operations on down the processing

line. Initial reports from one of the processors that installed the electrical stimulation suggests that their reprocessing was reduced by 50% due to birds defecating during bleed.

OBJECTIVE B: Determine whether mixed scalding and picking affects carcass microbiology.

PROGRESS B: Some years ago it was established that simultaneous scalding and defeathering could significantly lower carcass bacterial levels. The effect of defeathering carcasses between the tanks of a multiple-tank scalder was tested in these experiments, with the idea that bacterial contamination caused by defeathering equipment might be mitigated by several subsequent dips in hot scald water. Contrary to expectations, initial trials have shown bacterial levels on treatment carcasses were not lower than on conventionally scalded and picked control carcasses.

IMPACT/TECH TRANSFER B: This work could lead to innovative modifications to the scalding and picking procedures for broiler carcasses. Adjustments to the scalder temperature and duration of scald times in the various scald tanks could lead to the reduction or elimination of cross-contamination now associated with standard poultry defeathering equipment.

OBJECTIVE C: Modify the computer model of bacterial mixing in poultry scald water to focus on potential for cross-contamination.

PROGRESS C: Previous computer modeling of bacterial mixing in scald water was a mass-action calculation using large numbers of bacteria. The model has been converted to use integer numbers of bacteria to examine the effects of cross-contamination between carcasses by bacteria such as salmonellae which are present in scald water at very low levels compared to aerobes or coliforms. Actual salmonellae levels in a commercial scald tank will be used to determine the likelihood of cross-contamination.

IMPACT/TECH TRANSFER C: By understanding the relationship of the number of organisms in the scalder with the movement and death rate of these organisms, accurate predictions could be made for the risk of further carcass cross-contamination due to the scalding procedures.

OBJECTIVE D: Develop methods to remove feces from the cloaca before defeathering, evaluate feed withdrawal on viscera weight and shear force and develop alternative methods of crop removal to minimize ingesta spillage and carcass microbial contamination.

PROGRESS D: Previous work showed that inserting cloth plugs in the cloaca before scalding could reduce some kinds of bacterial contamination on New York dressed carcasses by more than 80%, but on-line plugging is not feasible under current processing conditions. This work evaluated the microbiological status of New York dressed carcasses after using focused water sprays to clean the cloaca and colon before scalding under conditions that could be duplicated at commercial line speeds. Results showed that cloacal spraying did affect carcass bacterial levels,

but not always beneficially. Escape of spray water remaining in the colon was a problem during defeathering, and dissection showed that there was considerable post-mortem peristalsis and continued movement of feces in the lower gut even after successful removal of feces by spraying. The duration of feed withdrawal significantly reduced viscera weight, liver weight, intestinal diameter, and increased gall bladder length. The length of feed withdrawal (0 to 24 hours) did not affect intestinal shear strength. Liver color with longer feed withdrawal was darker, less red, and less yellow.

Initial trials determined a consistent breakpoint of the esophageal tract, at or within the crop, when pulling force was applied at the head. This breakpoint was consistent for tracts pulled *in situ* or when the entire tract was excised from the carcass prior to pulling. The force recorded at breakpoint for excised tracts was 1/3 less than those not excised, although the spinal column was severed at the shoulders. The difference in force at breakpoint indicates that neck skin and connective tissue adhesions to the esophagus significantly increased resistance to pull. Preliminary results indicated that the crop and its contents can be removed intact when the esophagus is severed within the thoracic cavity between the crop and the proventriculus and the spinal column severed at the shoulders. Implementation of an esophageal cut is adaptable to the recently introduced poultry evisceration systems. Analysis of crop pH revealed a consistent increase with longer feed withdrawal periods which may affect microbial growth and recovery.

IMPACT/TECH TRANSFER D: This work could lead to methodology for the reduction or elimination of fecal contamination post scald thus reducing the contamination of the carcasses with enteric pathogens associated with fecal material such as *Salmonellae* and *Campylobacter*.

These results contradict notions that longer feed withdrawal periods result in more tear prone intestines due to reduced rigidity. Therefore, the focus of feed withdrawal programs should be on cooping broilers at 4 hours off feed and transporting to the plant for processing after an additional 6 hours. Apparently only bile spillage would be associated with extended feed withdrawal periods. Therefore, extending feed withdrawal periods for broilers beyond 10 hours to further reduce potential of carcass contamination from intestinal spillage may be an improved standard operating procedure under HACCP.

Development of a crop removal process that consistently extracts the crop intact will benefit poultry processors and the consumer by reducing the internal and external surface contamination that occurs with current automated methods of crop removal. Initial contact with processing equipment manufacturer Stork Gameco has resulted in the promise of a loan of manually operated evisceration system to determine if the modified crop removal process will significantly reduce carcass microbial contamination.

OBJECTIVE E: Acid/ Base dips of New York dressed carcasses.

PROGRESS E: The use of low and high pH dips for various times (10, 20, 40, and 60 sec) and temperatures (20 and 40 C) was determined both alone and in tandem on New York dressed carcasses. Carcasses were dipped in solutions at either pH <3 or >12 for times up to one minute. Treatments were singular dips or tandem dips from high to low pH and low to high pH.

Carcasses were processed under commercial conditions and the vent plugged immediately post pick. After picking, carcasses were submerged in the treatment solutions, rinsed with tap water , and microbiologically sampled using the whole carcass rinse. Total aerobes were not significantly reduced, but coliforms and generic E-Coli were reduced by almost a $\log_{10}$ when the acid treatment was last. Microbial counts did not vary due to the temperature treatment.

IMPACT/TECH TRANSFER E: The use of an acid dip in tandem with trisodium phosphate could prove to be an effective intervention step in an overall HACCP plan for commercial processors. The delivery of a safer product to the consumer would be the primary impact and concern for the poultry processors. Further research into time/temperature treatments using the acid/base treatments will be conducted to find the optimum treatment conditions.

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SPECTRAL RADIOMETRY AS AN ON-LINE INSPECTION TOOL

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CRIS Number: **1270-42440-001**
FSIS Code: **B6II01**
CRIS Completion Date: **January, 1998**

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OBJECTIVE A: Develop a low cost, real-time automated system for the on-line detection of wholesome and unwholesome poultry carcasses (as defined by the Food Safety Inspection Service) in slaughter plants.

PROGRESS A: We have developed improved algorithms for on-line classification of wholesome and unwholesome poultry carcasses using the visible/near-infrared (Vis/NIR) spectrophotometer. We applied principal component analysis (PCA) technique to spectral reflectance data to reduce the number of input nodes to neural network classifiers. We found that, using PCA pretreatment of input data, the number of input nodes for a neural network could be reduced without compromising its accuracy. We also found, contrary to earlier conclusions, that sensing moving poultry carcasses without room light greatly improved the classification accuracies, compared to sensing in room light. In fact, when sensing in a dark environment, the classification models with PCA pretreatment achieved 100% accuracies for training, validating, and testing at both 60 and 90 birds/min shackle speeds. But sensing in room light, the best model had an accuracy of 95.8% at a shackle speed of 60 birds/min with PCA pretreatment and 96.2% without PCA pretreatment. For a shackle speed of 90 birds/min, the best model for sensing in room light had 96.8% accuracy with PCA pretreatment and 95.6% without PCA pretreatment. This research showed that PCA was a powerful technique in extracting important features of input data. It reduced the number of input nodes to the neural network classifiers, and in most cases improved the classification accuracy. The models with PCA pretreatment also required fewer training samples and reduced training time. This research also showed that when sensing carcasses in a dark environment using the Vis/NIR spectrophotometer system, wholesome and unwholesome were classified with 100% accuracy.

We also have tested the on-line poultry inspection multispectral imaging system for robustness and real-time operation. Two cameras with 540 nm and 700 nm filters were used to capture the front of the bird and two cameras were used to capture the back of the bird. The speed of camera images captured and processed by the computer was about 60 birds per minute. The discriminant functions for identifying unwholesome (septicemic and cadaver) carcasses were developed using linear and quadratic covariance matrix analysis methods. The accuracy of the quadratic discriminant models, expressed in rates of correct classification, was 93.3% for the identification of septicemic and 95.8% for cadaver carcasses. This occurred when textural features from the spectral images scanned at the wavelength of 540 and 700 nm were utilized.

The linear discriminant model was useful for the classification of wholesome carcasses (96.2% accuracy) and quadratic discriminant model was useful for the classification of unwholesome carcasses (97.1% accuracy).

The ISL pilot-scale instrumental inspection system for poultry carcasses has been tested for process automation. This pilot-scale system can operate speeds up to 100 birds per minute. Presently, both of the Vis/NIR system and the spectral imaging system can operate up to 100 birds per minute. Three high-speed personal computers and one high-speed spectrophotometer, 10 times faster than the current system, have been acquired and tested for implementation. As a result of higher processing speeds, the speed of instrumental inspection could be greatly increased. An industrial prototype of the ISL poultry inspection system has also been assembled and is ready for an in-plant testing for maintenance requirements, robustness, and easiness of on-line calibration in an actual processing plant environment.

IMPACT/TECH TRANSFER A: A brief review on the ISL machine vision system for on-line, real-time inspection of poultry carcasses was presented to managers of Allen Family Food, Inc. in Cordova, MD and to the managers of Townsend, Inc. (about 20 managers attended). A talk on the development of the ISL machine vision poultry inspection system was presented to the U.S. Poultry and Egg Association and FSIS staff. Two engineers from the Agriculture and Agri-Food Canada came to view the demonstration of the ISL pilot-scale machine vision system for on-line inspection of poultry carcasses. A review and demonstration of the ISL machine vision poultry inspection system was presented to Dr. Arshad Hussain, Chief of Technology Assessment Branch, FSIS, and his staff.

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CONTROL OF PATHOGENS ON SURFACES OF POULTRY
(Chemical and Physical Techniques)

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OBJECTIVE A: Using chemical and physical techniques of surface modeling, identify and describe mechanisms of the attachment of pathogens to surfaces of poultry. (This research includes the surfaces of fruits and vegetables)

PROGRESS A: Infrared and Raman spectroscopies are being investigated as tools to determine the chemical nature of attachment of pathogens to food product surfaces. Raman micro-spectroscopy can analyze bacterial cells with 1 micron spatial resolution and 2 micron depth profiling in a non-destructive, non-invasive mode, thus allowing measurements to be carried out on biofilm components. In preliminary work, *Campylobacter* and *Salmonella* from cultures were examined to determine feasibility. Reference spectra were obtained from purified lipooligossacharide (LOS) components as possible representatives of outer membrane attachment molecules.

IMPACT/TECH TRANSFER A: The work contributes to a basic understanding of attachment phenomena for bacteria.

OBJECTIVE B: Using chemical and physical techniques, evaluate the effect of inhibitors and disinfectants on food product surfaces.

PROGRESS B: Biofilm formation on food surfaces may proceed through a mechanism involving chemical sensing of attached bacteria. In preliminary experiments to understand the phenomena of biofilm formation, we are examining *Campylobacter* spp. for the presence of quorum sensing molecules that are known to act as chemical signals for aggregation.

IMPACT/TECH TRANSFER B: New strategies for inhibiting surface biofilm formation may provide novel approaches to reducing transmission of pathogens through food processing operations.

OBJECTIVE C: Develop rapid methods for identifying pathogenic bacteria using laser and thermal desorption mass spectrometry and artificial neural network analysis.

PROGRESS C: A laser desorption time-of-flight mass spectrometer (MALDI-TOF) was installed and tested. The MALDI-TOF method analyzes phospholipids, proteins and other non-volatile substances by laser impact on samples immersed in a UV-absorbing matrix material, yielding molecular weight profiles of the components. Whole bacterial cells mixed with matrix material such as alpha-cyano-4-hydroxycinnamic acid dissolved in triflouroacetic acid (TFA)-isopropanol/water solution are non-viable, thus simplifying transfer into the mass spectrometer. Conditions are being optimized for *Salmonella* by varying matrix material, concentration and solvent composition. Output data have been successfully configured in spreadsheet format in preparation for neural network and principle component analysis. Automated analysis of 100-sample disks has been demonstrated.

IMPACT/TECH TRANSFER C: The research addresses the need for rapid, specific confirmatory analysis of pathogens at the genus strain and sub-strain level.

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ADHESION MECHANISMS OF HUMAN PATHOGENS TO SURFACES OF POULTRY

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CRIS Number: **5325-42000-022**
FSIS Code: **W1MC02**
CRIS Completion Date: **December, 1999**

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OBJECTIVE A: To identify and describe the molecular mechanisms of the attachment of pathogens to surfaces of poultry. (This research includes the surfaces of fruits and vegetables)

PROGRESS A: Pathogenic and non-pathogenic strains of *Campylobacter* and *Salmonella* species and *E. coli* have been acquired or isolated. A library of mouse monoclonal antibodies (MAbs) that bind *C. jejuni* and *C. coli* have been produced. MAbs characterized so far represent several different specificities, including antibodies that bind: (1) to all strains of *C. jejuni* and *C. coli*, (2) to *C. jejuni* only, (3) to specific isolates of *C. jejuni* or *coli*, or (4) to molecules undefined at present. The binding specificity of the antibodies is currently under study by immunoblot techniques. The antibodies will be used to develop better isolation and detection methods. Specific studies in progress include *in situ* identification of *C. jejuni* by immunohistochemistry and development of assays to measure attachment of *C. jejuni* to poultry tissues.

IMPACT/TECH TRANSFER A: MAbs will be used to develop assays for faster detection and identification of pathogens.

OBJECTIVE B: To identify new technology, including new compounds, that can interfere with the adhesion of pathogens to food product surfaces.

PROGRESS B: Studies of attachment of *C. jejuni* to poultry tissue surfaces to determine the molecular basis of attachment have begun. Initial approaches include fluorescence microscopy, thin layer chromatography and SDS-PAGE bacterial overlay assays, microwell assays of tissue extracts, and chemical analysis of adherence factors. Preliminary studies using a microwell assay for measuring inhibition of bacterial attachment have begun. This approach will permit testing of potential attachment inhibitors.

IMPACT/TECH TRANSFER B: Information on the mechanism of pathogen binding to food surfaces (e.g., individually, biofilms with other bacteria, exterior/interior surface, etc.), will help producers develop strategies to control viable pathogen contamination and test inhibitors of attachment, and/or compounds for controlling the amount of viable pathogen contamination.

PUBLICATIONS:

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TREATMENT AND REUSE OF WATER IN COMMERCIAL FOOD PROCESSING OPERATIONS

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OBJECTIVE A: Improve the efficiency of use of chlorine dioxide as a disinfectant by developing monitoring methods and rational management strategies to prevent excess use which is not only costly but presents a serious health hazard to workers.

PROGRESS A: Research to develop an analytical ion chromatographic method to directly determine chlorine dioxide and its disinfection products, e.g. chlorite and chlorate, was initiated. Chlorite and chlorate were separated and quantified. Chlorine dioxide was reduced to chlorite during chromatography. The reduction was demonstrated to be quantitative by the analysis of the chromatograms and UV spectra obtained by photodiode array detector and conductivity detector. The detection limits for chlorine dioxide, chlorite and chlorate were 0.1 ppm. Although it is not capable of distinguishing chlorine dioxide and chlorite, the method was more sensitive and reproducible than the commonly used amperometric titration method for the detection of chlorine dioxide residuals in food products. It was adopted for the determination of combined residuals of chlorine dioxide and chlorite in potatoes treated with gaseous chlorine dioxide to prevent bacterial spoilage during storage.

IMPACT/TECH TRANSFER A: Chlorine dioxide is a stronger bactericide than chlorine in food processing water. It does not form trihalomethane and has not been found to produce mutagens as chlorine does. Despite its bactericidal and toxicological advantages, chlorine dioxide has not been adopted generally by the food processing industry primarily because of a lack of an adequate monitoring method for the control of safe and efficient use. The method under development is direct, sensitive, and reproducible and may fulfill the need for an analytical procedure provided the conditions of the chromatography can be manipulated to reduce, elminate, or account for reduction of ClO_2 to chlorite .

Chlorine dioxide is an FDA and USDA-approved substitute for chlorine in poultry processing water. It has a wider acceptance than chlorine as a drinking water disinfectant in Europe. The use of chlorine dioxide may improve the safety and quality of processed poultry and increase their acceptance in the export market to countries objecting to chlorine-based disinfection.

OBJECTIVE B: Develop methods for the simultaneous disinfection and treatment of poultry chiller water and food process fluids to improve water quality for extended use.

PROGRESS B: This new research focus included an extensive review of the literature on flotation technology with particular attention to its application to the food industry. A bench-scale (2 liter capacity) continuous flotation device was designed and constructed. This device is the embodiment of a means for applying high efficiency flotation and simultaneous application of chemical disinfectant. This unit can control such independent parameters as flow rate, gas introduction rate, overflow fraction, liquid introduction and removal points, temperature and energy consumption. The device was applied to model protein solutions in various salt solutions using residence times and gas rates as independent variables. Removal of as much as 60% of dissolved protein was observed. The device was also applied to poultry chiller water using gas rates and temperature as independent variables. Suspended solids, turbidity and total organic carbon were monitored as well as the level of available chlorine in the effluent and the ability of the process to remove/disinfect aerobic and coliform bacteria. This device demonstrated the ability to simultaneously remove as much as 90% of suspended solids from poultry chiller water while effecting greater than a 4 to 7 log reduction of the natural microbial flora .

IMPACT/TECH TRANSFER B: Current data suggests a real potential for improving processing water quality and this potential will be expanded through additional experimentation. The process also may be used to treat the overflow poultry chiller water for flotation recovery of fat and proteins as by-products and to extend use of the treated water to reduce the discharge of wastewater and BOD to the environment.

PUBLICATIONS:

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MICROBIAL SAFETY CRITERIA FOR FOODS CONTACTING REUSE WATER IN FOOD PROCESSING PLANTS

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OBJECTIVE A: To compare the microbiological content of swine headmeat obtained by two deboning procedures.

PROGRESS A: The aerobic plate count (APC), coliforms, and *E. coli* content and *Salmonella* incidence and level of swine headmeat harvested by either the old or a new improved procedure were evaluated. In the new procedure we use 180°F water for washing and sanitizing work surfaces and equipment in contrast with 140°F water used in the old procedure; meat from the inside cheek is no longer harvested; and the amount of contact with the inside of the mouth is reduced. Based on 144 samples (72 by each procedure), the levels of APC, coliforms, and *E. coli* were 4.52±0.26, 2.37± 0.42, and 2.25±0.42 $\log_{10}$ cfu/g respectively, regardless of the procedure used for harvesting the meat. The incidence (27/72 *vs.* 28/72) and MPN (most probable number) levels (4 to 93 *vs.* 4 to 1100) of *Salmonella* [determined by the Bacteriological Analytical Manual (BAM) method] also were the same for headmeat obtained using either procedure. The BAM method detected a higher incidence of *Salmonella* (55/144) than other methods, PCR [38/144, BAX (DuPont/Qualicon PCR method)] or DNA hybridization (41/144; Gene-Trak). Time of harvesting during the processing day or site of origin of the headmeat (cheek *vs.* tongue *vs.* back of the head) had no effect on the incidence of *Salmonella.*

IMPACT/TECH TRANSFER A: The data obtained indicate that the levels of bacteria found on headmeat largely represent the flora and levels in and around the oral cavity of swine and suggest that procedures other than modifying the deboning techniques must be developed to reduce the levels of bacteria.

OBJECTIVE B: To develop a process to reduce the incidence of *Salmonella* and levels of bacteria found on swine headmeat.

PROGRESS B: A pilot plant scale washer and headmeat inoculated with *Salmonella* strains isolated during the incidence study were used to evaluate different headmeat treatments. A process was developed which combined a rapid hot water rinse [75-80°C] with cold shock [cooling to -2.2°C with the addition of salt]. This process reduced starting levels of *Salmonella*

from an MPN of *ca.* 1100/g to undetectable and substantially reduced the APC, coliform, and *E. coli* content of swine headmeat.

IMPACT/TECH TRANSFER B: Because of its color, swine headmeat is a desirable ingredient in various fresh sausages; however, its high microbial levels and its *Salmonella* content adversely affects the quality and potential safety of the finished products. The process developed reduces the microbiological content of this ingredient and can easily and readily be incorporated into the processing procedures currently used in the plant. This research was done under a Memorandum Of Understanding with a local pork processor, and the results have been shared with the company. The data obtained will be used, by the processor, as the basis for their petition to FSIS to utilize, in their plant, the process developed. The published results will be available for use by the entire industry.

OBJECTIVE C: To determine the efficacy of a change in sanitizer on the elimination/reduction of *Salmonella* and *E. coli* in hog hauling trailers; the efficacy of water washing of holding pens; and determine the identity of *Salmonella* isolates obtained from the trailers and hog holding pens.

PROGRESS C: Previous studies indicated that washing and sanitizing hog hauling trailers reduced *Salmonella* to undetectable levels and *E. coli* by about 2 log cycles. After changing the washing procedure, including the sanitizing agent, the results showed that *Salmonella* were reduced to undetectable and *E. coli* was reduced to $<1 \log_{10}$. Using this procedure, results indicated that washing and sanitizing hog hauling trailers between runs should eliminate *Salmonella* and thus contribute to breaking the transmission cycle of this bacterium. The hog holding pen floors were sampled before use, after use, and after washing with water using a pressurized nozzle. Results indicated that washing reduces bacterial level, but does not eliminate *Salmonella.* Washing does prevent the build up of bacterial levels. The *Salmonella* isolates were serotyped and results indicate that over 15 different serovars were isolated from the trailers and the same serotypes were isolated from hog pen floors. This suggests that the hog is the primary vehicle for *Salmonella* transmission.

IMPACT/TECH TRANSFER C: The results were provided to the sponsoring company and formed the scientific basis of changing their procedure for washing hauling trailers. In addition, the information was provided to the National Pork Producers Council and the USDA Technical Analysis Group on Food Transportation.

OBJECTIVE D: To study the biochemical, chemical, and physical nature of binding mechanisms of bacteria and meat surfaces using macromolecules in model systems.

PROGRESS D: A model system was developed by immobilizing whole cells of *E.coli* 0157:H7 to study the "real time" binding interactions of anti- *E. coli* immunoglobulin (IgG), Extracellular Matrix (ECM) components. Whole bacterial cells were immobilized on the sensor chip of a BIAcore and the interaction of these compounds were determined as single compounds or

BIAcore and the interaction of these compounds were determined as single compounds or mixtures. This biosensor analysis indicated binding with *E. coli* components. The glycosaminoglycans did not bind with the cell surface sensor. The bacterial sensor can be used to assess compounds that might inhibit bacterial attachment with connective tissues or ECMS.

IMPACT/TECH TRANSFER D: The bacterial sensor can be used to evaluate real-time interaction of surface molecules from animal foods and *E. coli*.

OBJECTIVE E: To study inhibition of collagen and collagen-laminin mixture attachment to *E.coli* 0157:H7.

PROGRESS E: A class of food additives of natural origin inhibited binding of ECM components to the *E. coli* surface as shown by the BIAcore. This group of compounds also mediated the detachment of ECM components which bound to the bacterial cell surface. BIAcore analysis also showed the relative inhibitory activity of these compounds. Cell aggregation assay was insensitive to determine the relative inhibitory effects but aggregation of bacterial cells and their inhibition was observed.

IMPACT/TECH TRANSFER E: This class of compounds appears to have the potential to detach *E. coli* from meat and poultry surfaces.

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Part III. POST SLAUGHTER PATHOGEN MODELING AND CONTROL

DEVELOPMENT OF MICROBIAL MODELING COMPONENTS FOR USE IN RISK ASSESSMENTS

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OBJECTIVE A: Create and distribute a new version of the Pathogen Modeling Program

PROGRESS A: Version 5.0 was released in March 1997. It is now a Windows program and can be downloaded through the Internet. A major improvement in the models was to calculate confidence intervals for the various predictions. This gives users a much better realization of the precision of the predictions. This version contains growth models for 8 foodborne pathogens, survival models for 4 pathogens and three clostridia models. Plans have been made for Version 5.1 which will be the first to have the first thermal death models and previously published irradiation inactivation models. In addition, it will have updates and additions to existing models and capability to do more complex graphing.

IMPACT/TECH TRANSFER A: Since March, this program has been requested by over 1120 people from regulatory agencies, industry and academia in 40 countries. It was selected as an example of ARS' research for the Congressional market basket.

OBJECTIVE B: Develop mathematical models of the behavior of foodborne pathogens.

PROGRESS B: *E. coli* O157:H7 survival: The survival times at pH 4.7 and 7.2 were affected by the organic acid anions. Survival times in BHI broths with lactic and acetic acids were the shortest, in citric and malic acid were longer, and in HCl-adjusted BHI the longest. Inducing acid resistance in stationary phase cells increased the thermal tolerance in the three strains tested (including Jack-in-the-Box and Odwalla strains). The acid adaptation also causing a shift from linear inactivation kinetics to non-linear (increased D-value and presence of a shoulder). This increased the time for a 5-log reduction by as much as four-fold. In apple juice at pH 3.6, differences in kinetics were observed, but the times for 5-logs inactivation were not affected as

much as in broths. Apple juice adjusted to pH 4.5 to 6.5 did lead to an increase in thermal resistance.

The variations in rates of growth, inactivation, and thermal inactivation and in virulence between 19 strains of *E. coli* O157:H7 from both outbreak and non-outbreak sources were found to be highly variable under identical circumstances. Four-fold ranges in survival and inactivation times and two-fold ranges in growth rates were typically observed.

The inactivation of the Salami strain of *E. coli* O157:H7 during the fermentation and drying of the pepperoni manufacturing process was found to be only 1.5 to 2.0 logs. This corroborated previous work in broths that simulated fermented meats. However, an increased thermal tolerance from the increased acidity that was observed in the broth studies was not observed in the actual meat product. The thermal death D-values of the *E. coli* O157:H7 that survived the fermentation process in pepperoni were determined to provide information needed for the addition of a heating period between the fermentation and drying steps.

Listeria monocytogenes: Cells were grown to exponential growth phase, stationary phase, starved in dilute broth, or desiccated at various temperatures and then transferred to broths at various temperatures, and the lag times observed. Exponential phase cells had the shortest lag times, stationary and starved were longer, and desiccated cells had the longest times. Within each cell group, long lag times were observed for cells grown at high temperatures (28-37°C) and transferred to low temperatures (4 - 8°C). Minimal lag times occurred with the smallest shifts between the temperature of the growth broth and the temperature of the new broth. Longer lag times were also observed for decreasing pHs jumps (pH 7 to 5) and for addition of salt (0.5 to 5%). Models were created that described the temperature shift data for the four cell types. Validation studies where the temperatures of broth cultures were shifted during continuous growth indicated the models are capable of predicting growth under these circumstances.

Studies were continued on the effect of stress conditions (temperature, sodium chloride, EDTA) on growth kinetics, cellular morphology, and proteins of *L. monocytogenes* Scott A. Initial results indicated that differences in total cellular protein patterns, examined by one-dimensional polyacrylamide gel electrophoresis, were mainly due to growth temperature. A study of cell surface proteins is in progress to provide more detailed information.

The effects and interaction of malic acid concentration (0.0 to 2.0 M) and pH (3.0 to 7.0) on the inactivation of *L. monocytogenes* at 28°C was studied in brain heart infusion broths. Inactivation rates were dependent on both acid concentration and pH. At lower pH values, lowering the pH or increasing malic acid concentration increased the rate of inactivation. At higher pH values, low concentrations of malic acid were somewhat protective as compared to HCl-adjusted controls. Overall, malic acid was a relatively benign organic acid; its antimicrobial characteristics are similar to those of citric acid and substantially less bactericidal than lactic or acetic acids.

Shegella flexneri: A previously developed model for aerobic growth of *S. flexneri* gave favorable predictions at 19 to 37°C, but not at temperatures below 19°C. Additional data were collected for growth at 10-19°C and combined with previously obtained data. Eight response surface models with terms for temperature, pH, sodium chloride, and sodium nitrite concentrations were calculated. Additional data on growth of *S. flexneri* inoculated into sterile foods were also obtained. Three of the new models were chosen for evaluation by comparison of predicted growth characteristics with those observed in foods. Improved agreement of observed and predicted values was obtained with the three new models. The second order regression models for lag time and generation time were included in the Pathogen Modeling Program Version 5.0.

Studies on inactivation (survival) of *S. flexneri* by chemical and environmental factors (temperature, acid, and salt) have been initiated.

IMPACT/TECH TRANSFER B: The D values for *E. coli* O157:H7 grown in certain acidic conditions were more heat resistant than those previously published. Appropriate regulatory agencies were informed that thermal resistance guidelines may need to be reexamined. The research on the survival during manufacture of fermented meats was a cooperative project with the National Food Centre, Dublin, Ireland and will have immediate consequences on the domestic and foreign reevaluation of the safety of these products.

The *L. monocytogenes* data provides the juice industry with basic information needed to understand and calculate how rapidly pathogenic microorganisms will die in malic acid containing juices. This information was forwarded to the Food and Drug Administration in relation to their current evaluation of the safety of apple cider/juice.

The variations between strains data demonstrate the need to know the relative characteristics of a particular strain that is used for setting control parameter values in a food process, a D-value, for example. The risk analysis will need to consider the consequences of a weak or hardy strain being present in the food.

The lag time information is needed to accurately model a series of food processing operations and storage periods as the food goes through the entire farm to consumer continuum. In many instances, the cells shift from the original growth rate to the new rate without reentering the lag phase. This information will be incorporated into the next generation of the Pathogen Modeling Program.

OBJECTIVE C: Develop concepts for developing and managing programs in quantitative microbial risk assessment and emerging pathogens.

PROGRESS C: Working with the National Advisory Committee on Microbiological Criteria for Foods, the International Commission for Microbiological Specifications for Foods, and the International Life Sciences Institute, scientific concepts were developed and organized related to

the application of quantitative microbial risk techniques to HACCP. A series of recommendations were developed and a risk assessment was provided to the Food Hygiene Committee of Codex Alimentarius on the suitability of establishing microbiological criteria for *Listeria monocytogenes* in foods in international trade.

Demonstration risk assessments were presented at numerous governmental and industry forums to advance the concepts of microbial risk assessment and to bring this technique to potential users. The need for better information on the quantitative distribution of microorganisms in raw ingredients, on the variation between strains and with growth-survival-thermal inactivation parameters, and on the human dose-response was advocated.

IMPACT/TECH TRANSFER C: Presentations on microbiological risk assessment techniques and concepts have been made to U.S. regulatory agencies and to Codex Alimentarius committees. The later initiated a reconsideration of its current standards for *Listeria monocytogenes*. Expertise from this project was requested by FSIS to participate on a team to create a *Salmonella enteritidis* risk assessment in eggs. The *Salmonella* risk assessment in eggs will be the first foodborne pathogen risk assessment specifically created for regulatory purposes.

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RISK MODELING TO IMPROVE THE MICROBIOLOGICAL SAFETY OF POULTRY PRODUCTS

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CRIS Number: **1935-42000-029**
FSIS Code: **E5MA04**
CRIS Completion Date: **December, 1997**

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OBJECTIVE A: To evaluate the DuPont RiboPrinter™, an automated ribotyping system, for its ability to identify and characterize isolates of *Salmonella* from broiler operations.

PROGRESS A: The version of the DuPont RiboPrinter™ used in this study produced *Eco*RI fingerprint patterns of genomic DNA using an *E. coli* probe to the ribosomal RNA operon. Identification was accomplished by band-matching of the DNA fingerprint patterns of *Salmonella* isolates from broiler operations to DNA fingerprint patterns of known strains of *Salmonella* in the RiboPrinter™ database. The database used in this study contained DNA fingerprint patterns for 94 strains of *Salmonella.* In addition to identification, the RiboPrinter™ characterized the isolates by assigning them to RiboGroups based on the similarity of their DNA fingerprint patterns. One hundred and twenty-two isolates of *Salmonella* representing 22 serotypes and 12 sources were used to evaluate the performance of the RiboPrinter™. We found that the RiboPrinter™ was limited in its ability to identify *Salmonella.* The RiboPrinter™ identified 9% of the isolates at the strain level, 29% at the serotype level, 39% at the genus level, and failed to identify 23%. In contrast, the ability of the RiboPrinter™ to discriminate between isolates by characterizing them into RiboGroups was very good. Compared to serotyping, which indicated that there were 22 different *Salmonella* among our 122 isolates, the RiboPrinter™ detected 31 different *Salmonella* by ribotyping.

IMPACT/TECH TRANSFER A: These results indicate that automated ribotyping is more discriminatory than serotyping but that it cannot replace serotyping because of its limited ability to identify *Salmonella.* Rather, serotyping and automated ribotyping should be used together in epidemiological investigations of *Salmonella* contamination. Results of this study were presented at the 1997 Annual Meeting of the Poultry Science Association, Athens, GA.

OBJECTIVE B: To survey isolates of *Salmonella* for their growth kinetics under different environmental conditions.

PROGRESS B: The variability that exists among strains of *Salmonella* for growth under different environmental conditions is not well-characterized. However, this knowledge is vital for estimating growth of *Salmonella* in poultry foods during temperature abuse. A study was

conducted in which 16 isolates of *Salmonella* from broiler operations were surveyed for their growth kinetics in laboratory medium under favorable growth conditions (i.e., 40°C, 0.5% NaCl, pH 6.0). Three to five growth curves were generated for each isolate. The growth curves were fit to a two-phase linear growth model that directly estimates lag time and growth rate. Lag time was affected by isolate ($P = 0.005$) and ranged from 0.73 to 1.38 h. Likewise, growth rate was affected by isolate ($P = 0.022$) and ranged from 0.78 to 0.94 log/ml/h.

IMPACT/TECH TRANSFER B: Results of this study indicate that growth of *Salmonella* under favorable growth conditions is not highly variable. Surveying *Salmonella* for their growth kinetics is an ongoing activity in our laboratory. By understanding the variability of growth kinetics that exists among strains of *Salmonella* our ability to assess the microbiological safety of poultry foods will be enhanced.

OBJECTIVE C: To develop simulation models that predict the impact of poultry production and processing on the probability of foodborne disease from poultry products.

PROGRESS C: With the advent of computer programs that perform simulations of models created in common spreadsheet programs, it is now possible to create computer models that predict the risk of foodborne disease from poultry products produced by specified farm to table scenarios. A farm to table simulation model for cooked chicken was developed. The model was constructed in an Excel spreadsheet and was simulated using @Risk. It simulates the production, processing, and consumption of 1,000 chickens. The top section of the model estimates pathogen load, whereas the bottom section of the model calculates the probability of foodborne disease in the human population. The top section of the model is divided into 28 nodes. Each node represents a unit operation/pathogen event in the farm to table continuum. Three types of pathogen events - contamination, reduction, and growth - occur in the model. Each pathogen event is defined by an incidence and extent. The extent of each pathogen event is defined by a triangular distribution consisting of a minimum, most likely, and maximum value. The bottom section of the model estimates food consumption and infectious dose and calculates dose consumed and probability of foodborne disease. During each iteration, @Risk randomly samples each triangular distribution based on its defined incidence and calculates the probability of foodborne disease. Thus, at the end of a simulation, @Risk has accumulated data on 1,000 iterations or chickens. The output from the model consists of a profile of pathogen incidence for the 1,000 chickens as they move through the farm to table continuum, a frequency histogram of the probability of foodborne disease in the human population, and pathogen load profiles for individual chickens as they move through the farm to table continuum. The published paper presents an example of how the model can be used to evaluate the impact of poultry production and processing on the microbiological safety of chicken.

IMPACT/TECH TRANSFER C: Results of the simulations performed to date indicate that the model developed is a very valuable tool for helping identify where in the farm to table continuum

pressure needs to be applied to improve the microbiological safety of poultry products. The model was presented at the 1997 Reciprocal Meats Conference in Ames, IA.

OBJECTIVE D: To determine the effect of physiological state on the growth kinetics of *Salmonella* and to develop mathematical models that predict growth of *Salmonella* as a function of physiological state and environmental factors.

PROGRESS D: The influence of the physiological state of *Salmonella* on their growth in food is not well understood and has not been modeled. Our first study in this area addressed the question of whether altering the pH at which *Salmonella* are grown to stationary phase would alter their subsequent growth kinetics. A full factorial design of previous pH (5.5 to 8.5), temperature (10 to 40°C), and pH (5 to 7) was used. Seventy-five growth curves were generated in laboratory medium with a pure culture of *Salmonella typhimurium*. The growth curves were fit to a two-phase linear growth model to obtain estimates of lag time (LT) and growth rate (GR). Quadratic polynomial models for LT and GR as a function of pre-pH, temperature, and pH were developed. Analysis of variance indicated that pre-pH had a minor effect ($P = 0.0397$) on LT and no effect on GR. The models were tested against LT and GR data not used to develop the models. The prediction error for the LT model was 9.2% for laboratory medium and 23.5% for cooked chicken burgers, whereas the prediction error for the GR model was 9.1% for laboratory medium and 22.1% for cooked chicken burgers.

IMPACT/TECH TRANSFER D: These data suggest that the previous growth pH does not alter the physiological state of *S. typhimurium*. Currently we are investigating and modeling the effect of the previous growth temperature on the subsequent growth kinetics of *Salmonella*. Understanding how and having models that predict the impact of physiological state on the growth of *Salmonella* will enhance our ability to make informed decisions regarding the microbiological safety of poultry foods. Results of this research were presented at the 1997 Review of ARS Poultry Research, Athens, GA.

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ASSURANCE OF MICROBIOLOGICAL SAFETY OF THERMALLY PROCESSED FOODS

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OBJECTIVE A: Define the heat treatment required to achieve a specified lethality for *Escherichia coli* O157:H7 in lean turkey, pork and lamb.

PROGRESS A: Thermal inactivation of a four-strain mixture of *E. coli* 0157:H7 was determined in lean ground turkey, pork, and lamb. Decimal reduction times (D-values) (time to inactivate 90% of the population) were calculated from the straight portion of the survival curves by plotting log of survival counts vs. their corresponding heating times. Also, regression lines were fitted to experimental data points that contributed to tailing or shouldering by a survival equation (model) developed by Dr. Whiting at ERRC using Gauss-Newton curve fitting program (ABACUS Software Program, ERRC, USDA, Philadelphia, PA) and two D-values were calculated, one for the major population and another for a subpopulation. The z-values were estimated by computing the linear regression of mean $\log_{10}$ D-values versus their corresponding heating temperatures. D-values, determined by linear regression, in turkey were 11.51, 3.59, 1.89, 0.81 and 0.29 min at 55, 57.5, 60, 62.5 and 65°C, respectively (z = 6.5C°). Using a survival model for non-linear survival curves, D-values in turkey ranged from 11.26 min at 55°C to 0.23 min at 65°C. When *E. coli* O157:H7 four-strain cocktail was heated in pork or lamb, D-values calculated by both approaches were similar at all temperatures. This shows that if lean ground turkey is used to validate the safety of a process for *E. coli* 0157:H7, that process will also be safe for lean pork and lamb.

IMPACT/TECH TRANSFER A: Thermal death time values from this study were sent to FSIS PPID/HACCP Division which used the information in the quantitative risk assessment document. Also, the findings will assist food processors in designing acceptance limits on critical control points that ensure safety against *E. coli* 0157:H7 in cooked turkey, pork, and lamb.

OBJECTIVE B: Determine the influence of pH, acidulant and growth temperature history of *L. monocytogenes* strain Scott A on the organism's heat resistance and fatty acid composition.

PROGRESS B: The organism was grown at 10°, 19°, or 37°C in brain heart infusion broth, acidified to pH 5.4 or 7 with either acetic or lactic acid, to late exponential phase (OD_{600nm} = 0.6).

Thermal death times at 60°C subsequently were determined using a submerged-coil heating apparatus. The surviving cell population was enumerated by spiral plating heated samples onto tryptic soy agar supplemented with 0.6% yeast extract and 1% sodium pyruvate. Decimal reduction times (D-values) were estimated by plotting a linear regression of $\log_{10}$ number of survivors versus heating time and taking the absolute value of the inverse slope. The thermal resistance of cells cultured at a particular temperature was significantly lower ($p < 0.05$) when lactic acid was used to acidify the medium to pH 5.4. Regardless of acid identity, D-values significantly decreased ($p < 0.05$) with increased growth temperature when the pH of the growth medium was 5.4 whereas D-values significantly increased ($p < 0.05$) with increased temperature at pH 7. At pH 5.4 adjusted with lactic acid, D-values were 1.30, 1.22, and 1.14 min at 10°, 19°, and 37°C, respectively. At pH 5.4 adjusted with acetic acid, *L. monocytogenes* failed to grow at 10°C; the D-values were 1.32 and 1.22 min when the organism was grown at 19° and 37°C, respectively. At pH 7, the values were 0.95, 1.12, and 1.28 min with lactic acid and 0.83, 0.93 and 1.11 min with acetic acid at 10°, 19°, and 37°C, respectively. The most abundant fatty acids (44-82 %) were branched chain saturated fatty acids (anteiso- and iso-C15:0 and iso-C17:0) regardless of pH, acidulant or growth temperature. However, there was an increase in C15:0 isomers at the expense of iso-C17:0 when the temperature of growth was lowered from 37°C to 10°C. While variable changes in longer chain fatty acids were found, the percentage of longer chain (C16 and C18) fatty acids was greatest when *L. monocytogenes* was grown at 37°C regardless of pH or acidulant.

IMPACT/TECH TRANSFER B: This work will have implications in thermal inactivation modeling studies; *L. monocytogenes* must be in its most resistant state for thermal death data acquisition. Also, food processors should take into account the environmental conditions during growth while designing thermal processes that ensure safety against the organism in ready-to-eat foods.

OBJECTIVE C: Define the time and temperature needed to inactivate *Escherichia coli* O157:H7 in undercooked beef and elucidate the mechanism for induced thermotolerance.

PROGRESS C: Duplicate beef gravy or ground beef samples inoculated with a cell culture suspension of a four strain cocktail of *E. coli* O157:H7 were subjected to sublethal heating at 46°C for 15 to 30 min, and then heated/cooked to a final internal temperature of 60°C. Survivor curves were fitted using a linear model that incorporated a lag period (T_L), and D-values and "time to a 4-D inactivation" (T_{4D}) were calculated. Heat shocking allowed the organism to survive longer than nonheat-shocked cells; the T_{4D} values at 60°C increased 1.56 and 1.50-fold in beef gravy and ground beef, respectively. In ground beef stored at 4°C, thermotolerance was lost after storage for 14 h. However, heat-shocked cells appeared to maintain their thermotolerance for at least 24 h in ground beef held at 15 or 28°C. A 25 min heat shock at 46°C in beef gravy increased the levels of two proteins with apparent molecular masses of 60 and 69 kDa. These two proteins were shown to be immunologically related to GroEL and DnaK, respectively.

IMPACT/TECH TRANSFER C: Food processors should take into account increased thermotolerance when designing thermal processes for meat products. The findings will be of immediate use to consumers and to the food industry and regulatory agencies to establish guidelines so that prophylactic measures are adopted and environmental stresses (such as heat) do not render bacteria better able to survive thermal processing procedures that normally would be considered adequate.

OBJECTIVE D: Determine the heat resistance of *Clostridium perfringens* vegetative cells in ground beef and turkey that included sodium pyrophosphate.

PROGRESS D: The heat resistance (55-65°C) of *Clostridium perfringens* vegetative cells in ground beef and turkey that included 0, 0.15 or 0.3% (w/w) sodium pyrophosphate (SPP) was assessed in bags heated using a water bath. The surviving cell population was assayed on tryptose-sulfite-cycloserine agar. The D-values in beef that included no SPP were 21.6, 10.2, 5.3, and 1.6 min at 55, 57.5, 60, 62.5°C, respectively; the values in turkey ranged from 17.5 min at 55°C to 1.3 min at 62.5°C. Addition of 0.15% SPP resulted in a concomitant decrease in heat resistance as evidenced by reduced bacterial D-values. The D-values in beef that included 0.15% SPP were 17.9, 9.4, 3.5, and 1.2 min at 55, 57.5, 60, and 62.5°C, respectively; the values in turkey ranged from 16.2 min at 55°C to 1.1 at 62.5°C. The heat resistance was further decreased when the SPP level in beef and turkey was increased to 0.3%. Heating such products to an internal temperature of 65°C for 1 min killed > 8 $\log_{10}$ cfu/g. The z-values in beef and turkey for all treatments were similar, ranging from 6.22 to 6.77°C.

IMPACT/TECH TRANSFER D: The findings, addressing FSIS needs, were sent to the FSIS PPID/HACCP Division which used the information to aid with the disposition of products subject to cooling deviations.

OBJECTIVE E: Validate a rapid technique for the specific detection of viable *Listeria monocytogenes* in cooked model beef gravy, based on reverse transcription-polymerase chain reaction (RT-PCR) technology.

PROGRESS E: Previously, we developed a method for the specific detection of viable *L. monocytogenes*, in broth culture, based on RT-PCR amplification of a portion of the iap mRNA of *L. monocytogenes*. Model beef gravy inoculated with about 7 $\log_{10}$ cfu/g was heated at 60°C using a submerged coil heating apparatus. Samples (0.6 ml) were removed at predetermined time intervals. Following a 1 hr enrichment incubation, total RNA was isolated and DNase-treated. A 318 bp fragment of the iap mRNA was amplified by RT-PCR and the amplified product detected by Southern hybridization to an internal digoxigenin-labeled probe. The RT-PCR assay was specific for *L. monocytogenes* and demonstrated a level of sensitivity in the range of greater than or equal to 10 cfu/ml in cooked beef gravy.

IMPACT/TECH TRANSFER E: The rapid diagnostic test could provide a potential tool for confirmation, by FSIS, of the presence of *L. monocytogenes* in cooked foods.

OBJECTIVE F: Define the time and temperature needed to ensure safety in relation to control of *Clostridium perfringens* during cooling of cooked products.

PROGRESS F: The effect of temperature (15-52.5°C) on the growth from a spore inoculum of a three strain mixture of *Clostridium perfringens* was determined in Trypticase-peptone-glucose-yeast extract broth. *C. perfringens* population densities were determined at appropriate intervals by plating on to tryptose-sulfite-cycloserine agar. Growth data were analyzed by the Gompertz equation; the gompertz B and M parameters were then used to calculate lag phase duration (LPD), exponential growth rate, generation time (GT), and maximum population density values. *C. perfringens* growth from spores was not observed at 15°C or 52.5°C in up to 3 weeks. The generation time was as short as 7.8 min at 45°C. A reduction in the temperature of incubation had an increasingly marked effect on both the lag and GT time. A mathematical model shown to be quite accurate for describing bacterial growth throughout the entire temperature range was used to fit the observed generation and lag time data; the model was capable of predicting spore outgrowth and multiplication at various temperatures.

IMPACT/TECH TRANSFER F: The findings, addressing FSIS needs, were sent to FSIS PPID/HACCP which used the information to aid with the disposition of products subject to cooling deviations.

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STRESS ADAPTATION AND VIRULENCE EXPRESSION OF BACTERIAL PATHOGENS IN FOOD ENVIRONMENTS

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OBJECTIVE A: Develop an effective process to eliminate *Listeria monocytogenes* from ready-to-eat meat and poultry.

PROGRESS A: While incidence of listeriosis has decreased in the U.S., continued isolation of *Listeria monocytogenes* from ready-to-eat foods indicates this microbial hazard remains a health risk, if contaminated products reach consumers. To solve the problem we determined that cold shock, prior to heating, reduces the thermal death time of *Listeria. L. monocytogenes* Scott A had a D_{60}-value of 1.27 min, compared to 1.0, 0.9, 0.9, and 0.9 min, for samples that were shifted to 15°, 10°, 5° and 0°C for 1 h, respectively, then heated to 60°C. Thermal death time was not restored to control levels after incubation for 3 h at 28°C. Cold shock duration (1-3 h) had a greater influence on thermal death time reduction than did temperature down shift amplitude (37°C to 15-0°C). Stationary phase *L. monocytogenes* was most responsive to the cold shock effect, log phase cells were least responsive, and lag phase cells were intermediate. The Z-value was reduced by 15% to 7.71°C, in cold shocked samples, compared to 8.84°C in control cells. *L. monocytogenes* V7 and *L. inocua* exhibited a 33% and 25%, decrease in thermal death time, respectively, after a 3 h cold shock at 0°C. No injury occurred from the cold shock, but did occur as a result of heating. Nearly full recovery of treated cells occurred in an anaerobic atmosphere. Thermal death time of *L. monocytogenes* Scott A was reduced by 25% on vacuum packaged frankfurter skins, after being cold shocked at 0°C, then heated at 60°C.

IMPACT/TECH TRANSFER A: The demonstration that temperature down shift of foods contaminated with *L. monocytogenes* resulted in lowered thermal death time indicates the potential of this phenomenon as an effective post-processing pathogen intervention strategy.

OBJECTIVE B: To develop a system for direct isolation of plasmid-bearing virulent serotypes of *Yersinia enterocolitica* from various food samples by a direct culture technique and by a multiplex PCR.

PROGRESS B: A procedure was developed for direct detection, isolation, and maintenance of different plasmid-bearing virulent strains of *Yersinia enterocolitica* (YEP+) representing five

serotypes - 0:3, O:8, O:5, O:27, O:13 and O:TAC - from a variety of foods. YEP+ strains representing five serotypes were simultaneously detected and isolated from enriched swab samples of artificially-contaminated pork chops, ground pork, cheese, and zucchini using Congo red binding and low calcium response tests. The method was also effective for isolating YEP+ strains from naturally-contaminated porcine tongues. Virulence of the strains isolated from these foods was confirmed by PCR, the expression of plasmid-associated phenotypes, and mouse pathogenicity. As low as 0.5 cfu of YEP+ per cm^2 of spiked food samples can be detected and the time required for isolation and confirmation is reduced from 6 days to 4 days with this new procedure. The advantages of the procedure include: (1) a single enrichment medium for a range of serotypes, (2) eliminates one day of enrichment and another for presumptive isolation, (3) uses a single medium (CR-BHO) for direct detection and isolation, and (4) preserves the virulence plasmid. A simplified method was developed for the direct application of multiplex PCR for screening different strains of YEP+ serotypes from the same food sources using centrifugation to prepare the enriched samples for multiplex PCR. The method was also effective in identifying YEP+ from naturally contaminated porcine tongues. The use of swabs eliminated the time-consuming DNA extraction step from food, inhibition of PCR from food-derived DNA, interference induced by background flora, and reduced the time needed for processing samples. The detection of other food pathogens should be feasible by this technique.

IMPACT/TECH TRANSFER B: This improved technology for screening and isolation of pathogenic *Yersinia enterocolitica* will be transferred to FSIS Microbiology Division through hands-on demonstration and a detailed written description.

OBJECTIVE C: To develop a method for detection of *E. coli* O157:H7 and *Salmonella* simultaneously in various types of samples using a multiplex PCR approach.

PROGRESS C: *E. coli* O157:H7 and *Salmonella* are two major food-borne pathogens which can be found as contaminating organisms on similar types of foods and other samples. A multiplex PCR assay was developed for simultaneous detection of *E. coli* O157:H7 and *Salmonella* spp. in enrichment cultures of ground beef, apple cider, cattle feces, and beef carcass wash water. The samples were inoculated with both *E. coli* O157:H7 and *S. typhimurium* at various bacterial levels. Following enrichment culturing for 20-24 hours at 37°C in modified EC broth containing novobiocin, the samples were processed and tested by the multiplex PCR assay. Four sets of primers were employed in the PCR: primers for amplification of the *E. coli* O157:H7 *eaeA* gene, conserved sequences of stx_1 and stx_2 genes, and a plasmid DNA sequence and for amplification of a portion of the *Salmonella invA* gene. Four fragments of the expected sizes were amplified in a single reaction and visualized following agarose gel electrophoresis. The detection limit was in the range of 3-25 colony forming units of *E. coli* and *Salmonella* per 25 g of ground beef or cattle feces, per 25 ml of apple cider and per 300 ml of beef carcass wash water. The entire procedure including enrichment culturing can be completed in 1 and 1/2 days.

IMPACT/TECH TRANSFER C: The method allows for rapid simultaneous detection of *E. coli* O157:H7 and *Salmonella* in various types of samples. The technology will be transferred to FSIS Microbiology Division through a hands-on demonstration.

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OPTIMIZATION OF THE SAFETY, QUALITY, AND SHELF-LIFE OF IRRADIATED POULTRY AND RED MEAT

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FSIS Code: **E2MI02**
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OBJECTIVE A: To determine the fate of gamma irradiated *Listeria monocytogenes* during refrigerated storage on raw or cooked turkey breast meat.

PROGRESS A: The radiation resistance and ability of *Listeria monocytogenes* ATCC 7644, 15313, 43256, and 49594 to multiply on irradiated, air packed, refrigerated raw and cooked turkey breast meat nuggets (ca. 25 g) and ground turkey breast meat was investigated. Gamma-radiation D values for *L. monocytogenes* were significantly different on raw and cooked nuggets, 0.56 ± 0.03 kGy and 0.69 ± 0.03 kGy, respectively; however, the radiation resistances were not significantly different ($P \leq 0.05$) on raw and cooked ground turkey meat. High populations (~10^9 cfu/g) of *L. monocytogenes* declined during 14 days of storage at 4°C in both irradiated and non-irradiated samples of raw but not in cooked ground turkey breast meat. A moderate inoculum (~10^3 cfu/g) did not survive a radiation dose of 3 kGy. The cfu increased in cooked but not in raw samples of irradiated ground turkey stored at either 2 or 7°C for 21 days. The D value changed significantly from 0.70 ± 0.04 to 0.60 ± 0.02 kGy when the product was cooked to an internal temperature of 80°C before irradiation. Growth on either raw or cooked turkey meat did not alter the radiation resistance of *L. monocytogenes*. Analyses were performed for pH, a_w, moisture, and reducing potential of raw and cooked turkey meat and of the pH, amino acid profile, thiamine, and riboflavin in aqueous extracts of raw and cooked turkey meats without identification of the factor or factors involved in differences in the survival and multiplication on raw and cooked meat.

IMPACT/TECH TRANSFER A: Results provide guidance to industry, FSIS, and FDA on the effectiveness of gamma irradiation for the control of *L. monocytogenes* that may contaminate precooked poultry products and multiply during refrigerated storage before sell or in the home.

OBJECTIVE B: To identify the radiolytic products of riboflavin.

PROGRESS B: Riboflavin was irradiated under various conditions of temperature, pH and the presence of organic compounds by light-catalyzed and gamma radiation oxidation. Both oxidation processes are radical reactions, and the products were found to be the same. Light catalysis resulted in the formation of two chromatographically distinct forms of both lumichrome

and lumiflavin, but gamma irradiation resulted in only one of the forms of lumichrome. Lumiflavin and lumichrome occur naturally in a number of food stuffs.

IMPACT/TECH TRANSFER B: Basic knowledge of radiation chemistry with application to the analysis of riboflavin.

OBJECTIVE C: Evaluate methods for indentification of irradiated meats using 1) supercritical fluid extraction (SFE) of hydrocarbons as an alternative to solvent extraction and 2) rapid micro column chromatographic methods for the separation of hydrocarbons.

PROGRESS C: SFE. Ionizing radiation is used to eliminate foodborne pathogens and to extend shelf-life of meats and poultry. Changes in volatile hydrocarbons following irradiation are considered to be indicators of irradiated meat and poultry. A method is described for the isolation and analysis of volatile hydrocarbons from irradiated meats using supercritical fluid extraction and subsequent identification and quantitation of individual hydrocarbons by gas chromatography with a mass selection detector (GC/MSD). Supercritical carbon dioxide at 175 bar and 40°C extracted the hydrocarbon fraction from total meat lipids within 20 min. The presence of radiolytic hydrocarbons, as determined by GC/MSD, corresponded to the radiation dose. Besides being faster, this method has the advantage of reduced solvent consumption when compared to current methods for determining if a meat or meat product has been irradiated.

Micro Column Chromatography. A small column chromatographic procedure was developed to isolate 1,7-hexadecadiene and 8-heptacecene, used as markers to detect beef, pork, or chicken treated with ionizing radiation. The hydrocarbons were isolated from the fat of these species using a Pasteur pipette packed with 3.5 g activated alumina. The relatively polar orange-yellow hydrocarbon, beta-carotene, was used to visually indicate elution of these non-chromogenic hydrocarbons. The small column replaced a standard sized chromatography column (approximately 2 cm x 20 cm) packed with 20 g activated Florisil. This use of the small column substantially reduced the amount of tissue required, organic solvent used, and the total time needed to isolate and prepare the samples for subsequent gas chromatographic analysis. Adding the highly colored beta-carotene negates the need for stringent control of the activity of the adsorbent, since the hydrocarbons always precede the carotene, and it is clear when they have been eluted completely off the column.

IMPACT/TECH TRANSFER C: Methods suitable for regulatory purposes have been developed for the identification of irradiated meat and poultry products. The techniques will reduce solvent use, cost and amount of time required by regulatory agencies to identify irradiated meats.

OBJECTIVE D: To compare the combined effects of irradiation and air, vacuum, and modified atmosphere packaging on the survival of *Listeria monocytogenes* on turkey meat when stored at refrigeration temperatures.

PROGRESS D: Several factors influenced the effectiveness of combining irradiation with modified atmosphere packaging (MAP) to control and/or eliminate *L. monocytogenes* during extended storage of turkey meat at refrigeration temperature. When inoculated ground turkey meat was packaged under mixtures of nitrogen and carbon dioxide and irradiated with gamma radiation doses of 0 to 3.0 kGy, greater lethality was found with 100% carbon dioxide than with 100% nitrogen. The radiation resistance of *L. monocytogenes* at 7°C on turkey was not only delayed by a MAP of 50% CO_2 and 50% N_2 compared to aerobic packaging, but was toxic to irradiated cells. The effects of MAP mixtures of 25, 50, and 75% CO_2 and N_2 were compared to aerobic and vacuum packed turkey inoculated with 5×10^3 cfu/g. The samples were irradiated to doses of 0, 0.5, 1.0, 1.5, 2.0, and 2.5 kGy and stored at 7°C for 28 days. Increased lethality was noted with increased CO_2 content. Only in a MAP containing 25% CO_2 did *L. monocytogenes* survive refrigerated storage for 21 days.

IMPACT/TECH TRANSFER D: This information is expected to be of value to regulatory agencies and to poultry and meat processors for the packaging of an irradiated product.

OBJECTIVE E: To determine the effect of pH and acid tolerance on radiation resistance of enterohemorrhagic *Escherichia coli.*

PROGRESS E: Work characterizing the effect of pH (4.0 to 5.5) and pH-dependent stationary phase acid tolerance was completed for a set of seven enterohemorrhagic and one non-enterohemorrhagic strains of *Escherichia coli.* Cells grown in acidogenic (final pH 4.7) and non-acidogenic (final pH 7.2) media were subsequently irradiated at 2°C. Induction of acid tolerance consistently increased radiation resistance, increasing D-values by as much as two-fold. Decreasing pH increased somewhat the radiation sensitivity of cells grown in the non-acidogenic media, but this effect was not as apparent in the acid tolerant cultures. There was considerable strain variability in the radiation resistance of *E. coli* isolates. The results indicate that low dose irradiation processing could be used to control *E. coli* O157:H7 in acidic foods such as fermented meats and apple cider, but that prior growth conditions need to be considered to accurately estimate the doses required.

IMPACT/TECH TRANSFER E: Provided information that is needed to accurately determine the irradiation doses needed to inactivate enterohemorrhagic *E. coli.*

OBJECTIVE F: To determine the effect of acid identity and the induction of acid tolerance on the radiation resistance of enterohemorrhagic *Escherichia coli.*

PROGRESS F: The effect of acid identity on the radiation resistance of the "Jack-in-the-Box" strain of *E. coli* O157:H7 was evaluated using BHI containing 0.5% lactic, acetic, or citric that was adjusted to pH 4.0 to 5.5 using HCl. Cells grown in acidogenic and non-acidogenic media were used to evaluate the cross-protection effects that result from the induction of pH-dependent, stationary phase acid resistance. These data were then compared against earlier studies with

BHI adjusted to these pH values using HCl only. With the cells not induced to acid resistance, the differences in irradiation D-values among the acids were small immediately after irradiation, and did not change after 7 days of storage at 2°C. However, substantial differences were observed using the cells induced to acid resistance; acetic acid reduced radiation resistance by approximately 1/3. Differences among the acids were further exaggerated by subsequent refrigerated storage.

IMPACT/TECH TRANSFER F: These data provide basic information needed to accurately calculate the low-dose irradiation processes that would be needed to pasteurize acidic foods such as fermented meats and apple cider.

OBJECTIVE G: To identify the effects of gamma irradiation on soaker pads in the presence of an aqueous food simulant.

PROGRESS G: In this study, a soaker pad commonly used in meat/poultry packaging composed of cellulose and polyester (PE) was put into bottles containing 10% ethanol food simulant. The samples were exposed to gamma radiation doses of 0 and 2.3 kGy. Polar compounds migrating from cellulose of PE were analyzed using HPLC. Mono/disaccharides at concentrations of 16-40 ppb and 63-143 ppb migrated from the irradiated soaker pad into 10% ethanol. Qualitative differences were observed in the HPLC chromatograms of PE extracts before and after irradiation.

IMPACT/TECH TRANSFER G: Work has been initiated with commercial firms, the FDA laboratory, the National Center for Food Safety and Technology, and ARS to develop the necessary information to obtain FDA approval of this and similar materials for food irradiation.

OBJECTIVE H: To evaluate PCR and DNA hybridization protocols for detection of enterotoxigenic *Clostridium perfringens* in irradiated beef.

PROGRESS H: The inability to differentiate between live and dead bacteria creates a problem with the use of some rapid molecular detection methods for harmful bacteria in foods. The erroneous indication that dead cells are alive could inappropriately result in regulatory action or self-imposed economic loss, by the food industry. Here, techniques, based on the detection of bacterial DNA, were compared with the conventional plate count method. All methods were examined in raw and cooked ground beef patties containing killed cells, as measured by the plate count method. The colony hybridization method showed reliable results, as demonstrated by the negative results under conditions where no cells were detected by the conventional method.

IMPACT/TECH TRANSFER H: The study provides information to the food industry and regulatory laboratories pertaining to the reliability of DNA-based detection methodologies applicable to food safety.

OBJECTIVE I: To determine the effect of pH-dependent, stationary phase acid resistance on the thermal resistance of enterohemorrhagic *Escherichia coli*.

PROGRESS I: The ability of pH-dependent, stationary phases acid resistance to "cross-protect" *E. coli* by increasing its thermal resistance was examined using three strains ("Jack-in-the-Box", "Odwalla", and A9124-C1) grown in acidogenic and non-acidogenic media. Cells were then transferred to pH 6.0 buffer and heated at 58°C using a submerged coil apparatus. Inducing acid resistance increased the thermal tolerance, causing a shift from linear inactivation kinetics to non-linear (increased D-value and the presence of a shoulder) . This resulted in an increase in the time to achieve a 5-D kill by as much as four-fold. Z-values for cells grown in acidogenic and non-acidogenic media were 4.7 and 4.3 degrees, respectively, over the range of 56° - 62°C. When this effect was examined in apple juice (pH 3.6), differences in the inactivation kinetics were noted but there was no difference in the time to a 5-D inactivation. Subsequent studies using pH-adjusted apple juice indicated the increased thermal resistance afforded by acid resistance induced cross-protection is influenced by pH; increased inactivation times occurred at pH 4.5 - 6.5, but not at 3.6.

IMPACT/TECH TRANSFER I: Comparison of D-values with those previously published for enterohemorrhagic *E. coli* indicated that current values may under estimate the pathogen's thermal resistance if cells grown under acidic conditions were transferred to a more neutral food (e.g., meat). Appropriate regulatory agencies were informed that thermal resistance guidelines *E. coli* O157:H7 may need to be reexamined.

OBJECTIVE J: Determine the sites on apples where *E. coli* O157:H7 are most likely to occur and how contamination is affected by apple temperature and determine the radiation dose needed to achieve a 5-D inactivation of enterohemorrhagic *E. coli* in apple cider.

PROGRESS J: Intact apples free of defects were dipped into cold (2°C) peptone water containing the "Odwalla" strain of *Escherichia coli* O157:H7 for 20 min. The temperature of the apples was either 2°C or room temperature (approximately 22°C). After air drying for 30 min, half of the apples were surface sanitized using a 0.2% bleach solution for 1 min followed by a tap water rinse for 1 min. The apples were then cut into peel, pulp, outer core, and inner core section using sanitized apple core knives. The sections were then assayed for *E. coli* levels. The greatest concentration was isolated from the outer core region (stem and blossom ends of the apple), with levels commonly exceeding 100,000 cfu/g. The skin was the second most contaminated reaching levels of 1000 to 10,000 cfu/g. Chlorine treatment decreased the levels on the surface by 2 to 3 log cycles, but was less effective at reducing the levels in the outer core region. Surface sanitization of the apples reduced the numbers of *E. coli* driven into the inner core and pulp during cutting. Conversely, placing warm apples into the cold peptone water tended to increase the penetration of the pathogen into the apples. Similar results were obtained with Yellow Delicious, Red Delicious, McIntosh, and Braeburn varieties of apples.

The radiation resistance of three strains ("Jack-in-the-Box," "Odwalla," and 932) of *E. coli* O157:H7 was determined initially in shelf-stable, clarified apple juice. Cross protection afforded by induction of pH-dependent, stationary phase previously observed in model systems was also observed in apple; prior growth in an acidogenic medium increased irradiation D-values by up to 150%. The "Odwalla" strain after acid resistance induction (the most resistant strain in its most resistant state) was then used to determine the dose needed to achieve a 5-D inactivation in a set of five different apple juices/ciders that ranged in clarity from an absorbance 0.04 to 2.01 and in pH from 3.5 to 3.9. Doses needed for a 5-D inactivation at 2°C ranged from 1.3 to 1.7 kGy, with doses increasing with increasing absorbance.

IMPACT/TECH TRANSFER J: Low dose irradiation is a viable intervention for inactivating *E. coli* O157:H7 in apple cider. These data were critical to developing Good Manufacturing Practices for the apple juice/cider industry, and though work is still underway, the results have been communicated to both the industry and the Food and Drug Administration.

OBJECTIVE K: To develop concepts for developing and managing programs in quantitative microbial risks assessment and emerging pathogens.

PROGRESS K: Working with the National Advisory Committee on Microbiological Criteria for Foods, the International Commission for Microbiological Specifications for Foods, and the International Life Sciences Institute, developed and organized scientific concepts related to the application of quantitative microbial risk techniques to HACCP and the development of programs for addressing the emergence of new foodborne pathogens. Developed series of recommendations and provided a risk assessment to the Food Hygiene Committee of Codex Alimentarius on the suitability of establishing a quantitative microbiological criteria for Listeria monocytogenes in foods in international trade.

IMPACT/TECH TRANSFER K: Presentations on microbiological risk assessment techniques and concepts have been made to US regulatory agencies and to Codex Alimentarius. The later initiated a reconsideration of its current standards for Listeria monocytogenes.

OBJECTIVE L: To develop a simple, inexpensive, aqueous method for screening poultry breast meat to determine if it has been irradiated.

PROGRESS L: Formaldehyde was observed to be generated in low ppm amounts in poultry breast tissue by low doses of gamma radiation. It was derivatized to a fluorescent compound and measured fluorimetrically. Differences between nonirradiated and irradiated (2 kGy) tissue stored at 4°C for up to 7 days or frozen for at least 4 weeks are large enough to allow correct identification of irradiated samples with an accuracy of 92%. The method is inapplicable to other poultry parts and to other animal tissue. Approximately 20 breasts can be screened per day per analyst.

IMPACT/TECH TRANSFER L: A very low cost, simple, rapid analytical method has been developed that is suitable for use by regulatory agencies to screen poultry breast tissues to determine if they have been irradiated.

OBJECTIVE M: To evaluate the potential of inactivating *Cyclospora cayetanensis* on fruits by irradiation.

PROGRESS M: This project was started as the result of an emergency request to ARS from FDA. The basis for concluding that it should be possible to inactivate *Cyclospora* by irradiation is that *Toxoplasma gondii* is very sensitive to ionizing radiation and has been inactivated by radiation doses of less than 0.5 kGy. T. gondii has the most resistant oocyst of the Coccidians. It also has the most efficient bioassay — 1 oocyst can be fatal. The mortality in mice is correlated with dose and it can be transmitted by the fecal oral route as well as by meat. Thus we believe that it is a good model for the investigation of *Cyclospora* which, unfortunately, does not have a suitable animal model. We (ARS and Univ. of Arizona) have completed four studies with *C. cayetanensis*. Typically we conducted irradiation inactivation studies with 50,000 sporulated and 50,000 unsporulated oocysts. We found that excystation occurred at doses thru 0.5 kGy. Sporulation occurred at doses up to 1.0 kGy though there was a dose dependent delay, morphological changes, and reduction in sporulation. *Toxoplasma gondii* also sporulated at doses up to 1.0 kGy but was demonstrated to be nonviable following doses ≥ 0.4 kGy. *T. gondii* oocysts irradiated at 0.4 kGy can excyst but do not multiply in the host. We are currently conducting additional research with *T. gondii* to confirm our conclusions.

IMPACT/TECH TRANSFER M: These results have been communicated both to industry and to the FDA.

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DEVELOPMENT OF MINIMALLY DEGRADATIVE PASTEURIZATION PROCESSES FOR LIQUID OR SOLID FOODS

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OBJECTIVE A: Develop surface pasteurization techniques to economically reduce microbial contamination on the surface of solid foods (e.g., poultry) without significant loss of product quality.

PROGRESS A: A prototype design to treat fresh whole broiler carcasses briefly with steam so that surface organisms are killed but no appreciable cooking of the meat occurs, was built, tested and patented. After brief heating by steam condensation, the carcass surface is quickly cooled below cooking temperature by re-evaporating the condensate back into vacuum. This novel approach depends on the lower activation energy of bacterial enzyme inactivation, contrasted to the higher activation energy of meat protein denaturation. This treatment is sufficiently rapid so that a single machine can serve 4000 birds per hour, which is the rate of a modern slaughter line. A two to four log reduction of applied *Listeria inocuans* have been achieved on fresh broilers without cooking by application of 145°C steam for 25 milliseconds. In the past year we developed a dual drive surface pasteurization unit for continuous operation; replaced the electronic motion control and actuation software system; installed a new product handling system; and scaled-up from drum sticks to whole chickens for optimization studies. We are conducting optimization studies in a batch automatic mode of processing and obtained an instrument to determine degree of cooking as a function of color and are developing the algorithms.

IMPACT/TECH TRANSFER A: The surface pasteurizer was demonstrated/tested for Perdue Farms using their chickens. We are awaiting feedback from Perdue on the results and possible interest. We are developing an agreement to test the system for extending the shelf life of catfish. Based on the success of the process on poultry, the system will be exploited to effect surface kills on intact fruits for insect eggs and molds and to surface heating of grains for disinfestation, decortication, and for germ separation.

OBJECTIVE B: Develop new electrical pasteurization technology applicable at lower temperatures for temperature sensitive liquid foods such as liquid egg.

PROGRESS B: For nearly 50 years, scientists have reported results indicating that Rf and microwaves seem to kill microorganisms which cannot be accounted for by thermal effects. Most scientists and equipment manufacturers dismiss the concept. We are investigating the possibility of developing a cold pasteurization process for liquid egg. Because of experimental difficulties, we chose to study and develop the process using low viscosity and low solids clear liquids such as water, juices, apple juice and cider, and beer. The work is in a food pilot plant so we use non-pathogens. We developed a double pipe/microwave system that appeared to give nonthermal destruction of non-pathogenic bacteria. We completed the study of a recycle, alternate feed process to simulate discrete, multiple passes through the microwave at low temperature. The results indicated microbiological destruction with no thermal damage; but, the phenomena is very elusive. Kills were observed with Pediococcus and yeast but no kills with non-pathogenic *E. coli*. We completed several experiments with liquid egg, whole, yolk, and white, but the results were ambiguous. There are apparently unidentified variables which are unknown. We are attempting to identify and isolate these variables using low viscosity, low solids liquids in a process to include high recirculation within the microwave to assure turbulent flow and no recycle.

IMPACT/TECH TRANSFER B: If and when we PROVE a non thermal effect, develop a process using appropriate organisms, such as molds and yeast in beer and an *E. coli* analog in cider. Exploit the process by applying it to liquid egg (whole egg, yolk, and egg white) using a *Salmonella* analog.

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DETECTION OF PATHOGENIC BACTERIA BY BIOSENSOR

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OBJECTIVES A: (1) determine the proper internal cellular physiological index for fluorescent detection of specific bacteria; to fully automate the magnetic field-assisted fluorescence microscopic bacterial enumeration system (2) identify the most favorable biochemical conditions to enhance the detectability of targeted bacteria by selected biosensor approaches, and develop new methods including electrochemical biosensors for bacteria detection., and (3) improve commercially available detection processes e.g., chemiluminescence and light-addressable potentiometric sensor, for bacteria detection.

PROGRESS A: Fluorescent Imaging of Bacteria: We used 4',6'-diamindino-2-phenylindole (DAPI), a nucleic acid-specific indicator to treat live *E. coli* O157:H7. Using conventional fluorescence measurement, we found that the intensity of labeled bacteria could be significantly enhanced by the presence of ATP in alkaline media and thus allowed a detection of about 3,000 bacteria per ml. This enhancement was not observed when ATP was replaced by other triphosphate nucleotides. Detailed characterization suggested that the interactions between ATP and labeled cellular components induced this enhancement. We then used antibody-coated magnetic beads to capture DAPI-labeled bacteria. More than 99% of captured bacteria could be aligned in a very small area by a magnet attached to microscope slides. This magnetic concentration step essentially reduced bacterial enumeration from a two-dimensional to a one-dimensional process. With the use of a sensitive Charged Coupled Devise (CCD) camera, we could easily detect the presence of about 10 cells. We have purchased an automatic 3-dimentional stage and a rapid recovering CCD camera which will automate this magnetic field-assisted fluorescence microscopic bacterial enumeration system.

Immunoelectrochemical Detection of Bacteria: Enzyme-linked immunomagnetic electrochemistry (ELIME) has been applied to the rapid detection of *E. coli* O157:H7 in buffered apple juice. The ELIME technique entails "sandwiching" bacterial analyte between antibody-coated magnetic beads and an alkaline phosphatase-conjugated antibody. The beads (with or without bound bacteria) were localized onto the surface of magnetized graphite ink electrodes in a multi-well plate format. The enzyme substrate, 1-napthyl phosphate, was added and conversion of substrate to an electroactive product was measured using electrochemical detection. Using this technique, detection of whole, live *E. coli* O157:H7 bacterial cells was achieved with a minimum detectable level of ca. 5×10^3 cells/ml in buffered saline or buffered

apple juice in an assay time of ca. 80 min. The ELIME response for the bacteria in either sampling medium was similar therefore indicating that the buffered apple juice did not contribute to any discernible sample matrix effects.

Light-Addressable Potentiometric Sensor Detection of Bacteria: An immuno-ligand assay (ILA) in conjunction with a light-addressable potentiometric sensor (LAPS) has been developed for the rapid detection of whole heat-killed or live *E. coli* O157:H7 cells in buffered saline. The ILA protocol consisted of "sandwiching" bacterial analyte between biotinylated and fluoresceinated antibodies, indirect enzyme labeling of the bacteria with urease-labeled anti-fluorescein antibody, and active capture of the immune complex at a biotinylated bovine serum albumin blocked nitrocellulose filter membrane with streptavidin. Using *E. coli* O157:H7, the efficiency of ILA was compared using various ratios of the biotinylated and fluoresceinated antibodies. Simultaneous addition of equimolar biotinylated and fluoresceinated antibodies effected optimal urease labeling of the bacteria in the ILA and the concentration of the antibodies was varied to achieve optimal LAPS detection of the bacteria. Using ILA with LAPS, a minimum detectable level of ca. 2.0×10^3 cells/ml of heat-killed or ca. 2.6×10^4 cells/ml of live, whole. *E. coli* O157:H7 bacteria was achieved in buffered saline in an assay time of ca. 45 and ca. 30 min, heat-killed and live bacteria respectively.

Filtration Capture and Immunoelectrochemical Detection of Bacteria: An improved detector for filtration capture and immunoelectrochemical detection of bacteria was developed using a porous electrode in contact with the filter to allow sample and reagent solutions to be delivered in a flowing system. This eliminated the need for manual assembly of the electrode and filter for each assay and allowed repetitive assays on a single filter/electrode. A computer-controlled fluid handling system was coupled to the detector to automate the previous assay protocol and provide new operating modes with enhanced background rejection and improved sensitivity.

Detection of Bacterial Culture Products: Using the ORIGEN (IGEN, Gaitherburg, MD) instrument, an electrochemi-luminescent bacterial detection system, the presence of antigenic material in the cell free supernatant of the medium used for growing *E. coli* O157:H7 was demonstrated. The material could pass through 0.2 micron filter but was retained by filters with a MW cutoff at 100 kD. Scanning EM indicated that the material, vesicular in shape, could bind to the anti-*E. Coli* O157:H7 antibody used for the ORIGEN procedure for the bacteria detection. Those vesicles, released from the outer membranes of the bacteria, could be used to determine whether specific food items have ever been exposed to the pathogenic bacteria.

IMPACT/TECH TRANSFER A: We have reported our progress to various scientific meetings including Biophysical Society, IFT, Pittsburgh Conference, etc. We also presented our research to manufacturers/processors in Apple Juice/Cider Conference (Wyndmoor, PA), to a Delegation from the Japanese Ministry of Health and Welfare, a Delegation from 22nd Food Sanitation Investigation, and representatives from Poly-Med, Inc, International Flavors and Fragrances, and Akers Laboratories. In addition to the existing Memorandum of Understanding (MOU) with the laboratory of the U.S. Air Force at Aberdeen, MD, we are developing a NSF Engineering

Research Center (ERC) proposal with the Department of Agriculture and Biological Engineering, Pennsylvania State University , University Park, PA for biosensor research.

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Part IV. RESIDUE DETECTION AND CHEMICAL ANALYSIS

ADVANCED TECHNOLOGIES FOR RESIDUE DETECTION (Supercritical Fluid Extraction and Chromotography, Microdialysis and Mass Spectrometry in Eggs and Other Food Animal Products)

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OBJECTIVE A: Develop a supercritical fluid extraction (SFE) method for the isolation of sulfonamides (SAs) from chicken liver at the regulatory tolerance level and apply SFE methodology to the detection of SAs and residues in beef, pork and eggs.

PROGRESS A: In the first part of this investigation, an SFE method was developed to recover three sulfonamides, sulfa-methazine(SMZ), sulfadimethoxine(SDX) and sulfaquinoxaline(SQX) from fortified chicken liver. Recoveries were 86%, 92% and 79%, respectively for the three SAs at the 0.10 ppm tolerance level. The current phase of this investigation is a comparison of the SFE method with the FSIS solvent-based method for SAs using chicken liver containing incurred SMZ. Results obtained thus far with the two methods indicate that recoveries from fortified and incurred tissue were about 50% by the FSIS method and 86% for the SFE method. Moreover, the FSIS method requires 40 ml of solvent compared to 2 ml by SFE. This method has now been applied to beef, pork and eggs.

Beef and Pork: Recoveries of SMZ, SDX, and SQX from fortified beef and pork tissues by this method were significantly lower than those obtained from chicken liver. These results are attributed, in part, to the differences in binding characteristics of the drugs with the different tissue matrices. To overcome this problem, various non-organic solvent modifiers such as water, acids, and phenylbutazone were added to the samples prior to SFE, in an attempt to reduce the effect of binding interactions. Addition of water to pork tissues fortified at the 0.10 ppm level gave improved recoveries of 76, 79 and 51% for SMZ, SDX and SQX, respectively. To achieve similar improvements in recoveries from beef tissues, fortified at the same level, 0.5 ml of phenylbutazone (20 µl/ml in water) was needed. This combination gave recoveries of 82, 89 and 61% for the three SAs, which approximated the recoveries obtained by the SFE method for fortified chicken liver.

Eggs: In the last report, we described the SFE conditions required for the effective extraction of three sulfonamides, SMZ, SDM, SQX, from eggs fortified at the 0.05, 0.10, 0.50 ppm levels. The SFE conditions were: SC-CO_2, without the use of a solvent modifier, 40°C oven, 10,000 psi (680 bar) with a flow rate of expanded gas of 3.0 L/min., to a total volume of 120 L. The SAs were trapped in-line on alumina, then eluted with a small amount of the HPLC mobile phase solvent system (phosphate buffer-MeOH), followed by HPLC separation on a C_{18} column and UV detection at 265 nm. Since these SAs were more readily extracted from eggs than noted in previous studies on meat tissue, this suggested a different matrix-SA interaction might be taking place. For this reason, egg laying hens were fed a single dose of SMZ to obtain eggs containing "normally incurred" SMZ. The feeding study was carried out in cooperation with CVM personnel. The eggs (n=21) were collected for up to 8 days post-feeding, then analyzed by both SFE and by a published solvent extraction procedure. SMZ levels ranged from 0.10 to 0.72 ppm by SFE and 0.10 to 0.78 ppm by solvent extraction; both gave an overall mean of 0.32 ppm. There was no statistical difference in the results obtained by the two methods. The CVs for SFE and solvent extraction were 3.9% and 5.0% respectively. The highest levels of SMZ were found 1-2 days after dosing.

IMPACT/TECH TRANSFER A: This study in chicken liver will be the first report on the SFE of incurred SAs from animal tissue. It is especially noteworthy since no SFE solvent modifier is used. The method will be transferred to FSIS and FDA for evaluation.

Beef and Pork: These results also demonstrate that it is now possible to achieve acceptable recoveries of the three SAs from beef and pork tissue using various non-organic solvent modifiers and slight modifications to the SFE procedure developed for poultry. These results will be of interest to FSIS and FDA laboratories who analyze tissue for SAs as part of their monitoring and surveillance programs. This method will be transferred to FSIS for further evaluation.

Eggs: These results indicate that the SFE of polar drugs, like SAs, heretofore thought not possible to extract by SC-CO_2 without the use of a solvent modifier, is now feasible at the higher extraction pressure of 680 bar. As a result, cleaner chromatograms are obtained without the necessity of additional clean-up of contaminants caused by coextraction by the solvent. The same FSIS laboratory carrying out SFE for SAs on meat tissue will now be able to perform these analyses on eggs. The method will be transferred to FSIS for evaluation.

OBJECTIVE B: To apply SFE for the extraction of amphenicol drug residues in tissue and eggs and determine the solubility of important veterinary pharmaceuticals in supercritical fluids (SFs).

PROGRESS B: Studies were initiated on the SFE of chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FAP) in fortified whole eggs without the use of a solvent modifier. Here, the phenicols are trapped in-line on Florisil, and after elution, analyzed by HPLC-UV detection at 225 and 280 nm. Further studies are planned with incurred chloramphenicol, and a comparison with an established method.

Solubility studies were conducted in different supercritical fluids (SFs) on the following drug classes: sulfonamides, β-agonists, and amphenicols. The measurements were performed in three solvents: carbon dioxide, fluoroform and 1,1,1,2-tetrafluoroethane (R 134a). The latter two SFs are environmentally acceptable alternatives to CO_2 for SFE. The three drug classes exhibited very low solubility in carbon dioxide over a pressure range of 170-470 bar. Similar results were obtained with the two fluorinated SFs, although other investigators have reported increased solubility for other analytes in these solvents.

IMPACT/TECH TRANSFER B: Amphenicols are prohibited from use in food producing animals, however their illegal use to combat serious infections still occurs. A multiresidue screening method for these potentially toxic compounds will allow the regulatory agencies to more effectively screen for these drugs.

The results of this investigation demonstrated that these alternative solvents provide no apparent increased benefits over carbon dioxide in the isolation of trace level veterinary pharmaceuticals from animal tissues. Additionally, these solvents are extremely expensive in comparison with carbon dioxide which would make their use impractical in regulatory laboratories. This information will be of importance to FSIS in developing SFE methods for use in their surveillance programs.

OBJECTIVE C: Apply SFE for the isolation of pesticide residues, dioxins and triazine (TRZ) herbicides in eggs.

PROGRESS C: We previously reported the effective SFE of 16 common organochlorine pesticides (OCPs) from fortified whole eggs. The OCPs included: aldrin, α-, β-, δ- and γ-BHCs, 4,4'-DDD, -DDE and -DDT, dieldrin, endosulfan I, II and sulfate, endrin, endrin aldehyde, heptachlor and heptachlor epoxide. Several of these OCPs have been reported to exhibit hormonal disruptive activity. More recent concerns have focussed on their possible role in the increased rate of breast cancer in Hawaii. The SFE conditions were similar to those employed for the SAs, except the OCPs were trapped off-line on a Florisil SPE cartridge, then analyzed by capillary GC-electron capture detector (ECD). In this case, off-line trapping was found to be superior to in-line trapping insofar as recovery, reproducibility and cleanliness of extract.

Eggs fortified at the 0.05 ppm level were extracted by SFE and the AOAC/FDA method for OCPs, and the results compared. All the recoveries were signicantly higher by SFE ranging from 81.8 to 108.3% and CVs <9.8%. Eggs containing normally incurred endosulfan I, one of the polar OCPs, were also analyzed by both methods. The results were comparable; however, the application of SFE had a number of advantages over the AOAC/FDA method.

Dioxins: Because of the successful application of SFE for OCPs in eggs, this work will be extended to include other organo-chlorine compounds, namely dioxins. This work has only recently been initiated and no progress is reported.

TRZs: Studies were initiated on the extraction of triazine (TRZ) herbicides from fortified eggs. Preliminary results suggest that several TRZs including: atrazine, cyanazine, simazine, propazine, prometryne, simetryn, ametryn, and prometon can be efficiently extracted by SFE without the use of a solvent modifier. Other TRZs, at residue levels, will be evaluated for their extractability by SFE so that a multiresidue approach will be possible.

IMPACT/TECH TRANSFER C: This SC-CO_2 method permits the rapid isolation of OCPs free of co-extracted lipids, without the time-consuming, solvent intensive extraction and concentration steps required by the currently employed techniques. This method effectively extracts many of the most important carcinogenic and environmentally persistent OCP contaminants. The method will be transferred to an FSIS laboratory for evaluation and use.

The recent events where dioxins were found in chicken tissue, eggs and catfish show the need for cost-effective, specific laboratory screening procedures. Results from these studies will lead to rapid regulatory implementation of these methods. SFE should also help in the confirmation of these compounds at the ppt level, since the need for extensive extract cleanup currently required may not be needed.

Under risk assessment guidelines it is expected that TRZs will need to be monitored in animal tissue and eggs. Their continued extensive use on animal feed crops, especially corn, is highly controversial since several members of this class exhibit carcinogenic and immunotoxic activity.

OBJECTIVE D: Investigate the use of automated on-line microdialysis for analyte sample preparation (analyte extraction and concentration) prior to HPLC determination.

PROGRESS D: The last report contained progress on the isolation of flumequine (FMQ) and oxolinic acid (OXO) from fortified chicken liver by on-line microdialysis using a Gilson ASTED system. Microdialysis was used in combination with reversed-phase HPLC and fluorescence detection (FLD). Recoveries (>90%) were achieved for these two fluoroquinolones (FQs). Work was expanded to include sarafloxacin (SAR) in an attempt to develop a multiresidue method for other members of this class of anti-bacterial agents now approved for use in poultry. Sarafloxacin had a different fluorescence absorption maximum wavelength than the other two FQs thereby requiring two HPLC runs to obtain the sensitivity required for residue determination. Because of this problem, a programmable FLD was purchased and compared to the single wavelength FLD. The programmable FLD permitted the detection of all three FQs with a single HPLC run. Employing standards, a limit of quantitation of 1.0 ppb and limit of detection of 0.2 ppb, were obtained. This was significantly superior to that obtained by a single wavelength FLD.

The effective application of this detector with microdialysis for the analysis of SAR, OXO, and FMQ in fortified chicken liver tissue was demonstrated. Recoveries of 85% were obtained over a range of 1-100 ppb. Chicken livers containing incurred SAR were obtained from a CVM

collaborator, who dosed the chickens at two levels for three consecutive days. SAR was isolated by microdialysis and by a solvent extraction procedure, then analyzed under the same HPLC-FLD conditions. The results obtained for the microdialysis method were comparable to those obtained by the other procedure. However, much cleaner chromatograms and greater sensitivity were obtained with the microdialysis method suggesting the application of this extraction technique is feasible for other members of this class of water soluble compounds.

IMPACT/TECH TRANSFER D: The use of a programmable fluorescence HPLC detector for the analysis of three FQs at the ppb level was demonstrated. This, combined with the advantages of using on-line microdialysis with its low organic solvent consumption, short sample processing time, small sample volume, automation and high sample throughput, makes this technique promising for routine monitoring by the regulatory agencies. There are plans to expand the scope of the method to include at least three other FQs prior to transfer to FSIS. FDA's CVM has also taken an interest in this approach.

OBJECTIVE E: Minimize the use of organic solvents by the application of mass-spectrometric techniques.

PROGRESS E: Both atmospheric pressure chemical ionization mass spectrometry (APCIMS) and electrospray mass spectrometry (ESMS) are being evaluated as in-line HPLC detection systems for oxolinic acid, a fluoroquinolone (FQ) antibacterial agent that has limited solubility in organic solvents. To date, reverse phase (C_{18}) HPLC with aqueous acetonitrile containing 0.1% ethanolamine and APCI detection has given the most promising results. The lower limit of detection (S/N>10:1) is in the order of 100 ng/ml. The sensitivity of the method is currently being optimized.

IMPACT/TECH TRANSFER E: Since this is a new approach for the analysis of FQs, at the ppb level, the impact is not yet apparent. However, demonstration of increased method sensitivity and selectivity in tissue extracts is desired before regulatory assessment.

OBJECTIVE F: Evaluate supercritical fluid chromatography (SFC) for the separation and detection of trace levels of pesticide and drug residues.

PROGRESS F: Under the cooperative agreement with Virginia Polytech., SFC studies were carried out using a chemiluminescence nitrogen detector (CLND) in combination with a packed HPLC column. Sulfonamides were used as the target analytes to determine detector sensitivity and selectivity. Several problems were encountered when using the CLND detector for this analyte class. The sensitivity of this commercial CLND was too low to measure the compounds at the levels needed for residue analysis in animal tissues. In addition, co-solvents needed for chromatographic separation of the drugs, such as methanol and water have a deleterious effect on the performance of the detector.

IMPACT/TECH TRANSFER F: On the basis of this investigation, it was concluded that SFC with a specific CLND did not offer a useful alternative to conventional HPLC for the analysis of trace level pharmaceuticals at this time. The agreement with Virginia Polytech was therefore concluded. This information will be useful to FSIS when evaluating new technologies for the detection of drug analytes since many of the drugs are nitrogen-containing compounds.

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METHODOLOGY DEVELOPMENT FOR RAPID ANALYSIS OF DRUG AND PESTICIDE RESIDUES IN FOOD ANIMAL PRODUCTS

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OBJECTIVE A: Develop, evaluate, and provide confirmatory testing of highly sensitive, inexpensive, monoclonal antibody-based, immunoassays useful for detection and quantitation of chemical residues in animal products and body fluids either on-the-farm, in-the processing plant, or for laboratory based analyses.

PROGRESS A: Development of monoclonal antibody based immunoassays are in progress for a number of compounds including: ceftiofur, fluoroquinolones, halofuginone, hygromycin B, 4,4'-dinitrocarbanilide, and various sulfa drugs (e.g., sulfamethazine). All of these have been identified by FSIS as being important for development of rapid immunoassays. The immunoassays for halofuginone, ceftiofur, and hygromycin B are the most complete.

Halofuginone (Hal). The halofuginone immunoassay detects residues in chicken livers between 50 and 200 ppb. It utilizes a simplified sample preparation method that does not require the use of organic solvents. This simplified method greatly reduces the sample preparation time as compared to the currently used FSIS HPLC method. The halofuginone immunoassay was formatted as a competition enzyme-linked immunosorbent assay (ELISA) and was used to evaluate spiked and incurred samples in comparison with HPLC analysis of the same samples by the FSIS Midwestern Laboratory (St. Louis, MO). There was good agreement in the results obtained by HPLC and with the ARS ELISA. However, in most cases the recovery was slightly better using the ELISA method vs. the HPLC method. The FSIS Midwestern Laboratory has agreed during 1997 to provide portions of each chicken liver sample, sent to them from the field for Halofuginone analysis, to our laboratory for comparison with ELISA analysis. Presently, we have analyzed 160 samples by the ARS ELISA and have similar results as those of the HPLC analyses at the FSIS Midwestern Laboratory. No violative samples (i.e., greater than 160 ng/g) have been found by either lab, however, some samples were positive for Halofuginone. We are looking at four cutoff levels of Hal: less than 50 ppb (nondetectable), greater than 50 ppb but less than 100 ppb, over 100 ppb but less than 160 ppb (this is considered positive), and 160 ppb or over (this is considered violative). Of the 160 samples analyzed by the two methods for comparison, the FSIS HPLC assay showed 149 samples had < 50 ppb, 11 with > 50 ppb, 2 with > 100 ppb, and 0 samples at 160 ppb or higher; and the ARS ELISA showed 138 samples had < 50 ppb, 22 with > 50 ppb, 3 with > 100 ppb, and no samples had 160 ppb or higher. To date, the ARS ELISA method compares well with the HPLC method and is performing as anticipated,

therefore it is able to indicate when a sample has a very high level of Hal or is violative. Such samples could then be reanalyzed by the ARS ELISA method and if again high amounts are detected , the samples can be analyzed by the more laborious FSIS HPLC method. In general, higher recoveries were obtained using the ELISA method, which may reflect the more simplified sample preparation method used with the ELISA. In this method, no organic solvents are needed for analysis. Thus, the ARS ELISA is a more environmentally friendly assay. At this point in the study, the data clearly demonstrate that the ARS ELISA method could be used as a screening method for the analysis of Halofuginone in chicken liver tissue.

Ceftiofur (Cef). Ceftiofur is an FDA approved veterinary cephalosporin antibiotic for the treatment of respiratory diseases in cattle, horses, and swine. We have developed a simple immunoassay for detection of ceftiofur and its metabolites in meats and milk. Our initial findings suggest that the cELISA detects not only ceftiofur but the major metabolite of ceftiofur, desfuroylceftiofur and conjugates of desfuroylceftiofur. Thus, unlike many HPLC methods which only measure parent or a specific metabolite, the cELISA results should be expressed as ceftiofur equivalents since it appears to detect metabolites and protein conjugates. Sample preparation for the ARS ELISA is simple and does not require the use of organic solvents. Meat samples are simply homogenized in buffer where as milk samples are simply diluted. For milk samples the average recovery observed was 100.5% with a range of 80 to 121%, and recoveries for beef kidney samples were similar. In order to facilitate transfer of this technology to FSIS, a detailed protocol describing the analysis of ceftiofur residues in meat samples was prepared and distributed, as well as the immunochemical reagents necessary to run the assay.

Hygromycin B (HyB). A monoclonal antibody capable of binding Hygromycin B was developed. This antibody was formatted into a rapid ELISA assay that has been used to analyze both spiked and incurred residues in pork kidney. Good correlations with a traditional chromatographic method were observed and the assay performed well.

Fluoroquinolones (Flu). Fluoroquinolones such as sarafloxacin are bactericidal antibiotics that have been used to control *E. coli* in poultry. A series of monoclonal antibodies to sarafloxacin have been developed and characterized, and studies are underway to format these antibodies into a simple immunoassay for analysis of chicken liver samples. Preliminary data from studies using spiked chicken liver samples suggest that the ARS immunoassay is capable of detecting sarafloxacin at levels as low as 10 ppb. Recently, incurred samples have been prepared and are presently being analyzed.

4,4'-Dinitrocarbanilide (44D). Nicarbazin is a drug used to prevent outbreaks of cecal and intestinal coccidiosis in chickens. It is comprised of a 1 to 1 molar ratio of 4,4'-dinitrocarbanilide (DNC) and 4,6-dimethyl-2-pyrimidinol. Only DNC is active and it is approved by FDA to determine the nicarbazin residues in chicken tissues. The established FSIS method determines the levels of DNC in nicarbazin. The tolerance level is 4.0 ppm in liver and muscle tissue, and it has an established withdrawal time of 4 days. Due to the difficulty of using derivatives of DNC to produce antibodies, we have synthesized mimics of DNC to be used as antigens and plate coating material. This work has been initiated with a CRADA partner (International Diagnostic System Corp.). Presently, mice have been immunized with our conjugates for the purpose of

producing monoclonal antibodies to DNC. In addition, IDS has used our conjugates to immunize rabbits for preparation of polyclonal antibodies to DNC.

Sulfa Drugs (SD). A series of monoclonal antibodies have been developed that bind various sulfa drugs, specifically, sulfamethazine, sulfadimethoxine, and sulfathiazole. These sulfa compounds were indicated as high priority by FSIS. To date we formatted these antibodies into a rapid ELISA. We have applied this ELISA to the analysis of chicken liver samples, both spiked samples and samples containing incurred residues. (The incurred residues were produced at our facility). Future objectives include completion of this evaluation and incorporation of these assays into a single ELISA capable of measuring each of these three commonly used sulfa drugs.

Other Compounds (OC). We continue our collaboration with Dr. Bruce Hammock, University of California-Davis in an effort to produce anti-dioxin monoclonal antibodies and analogs for dioxin. We have studied the response of an existing set of anti-dioxin monoclonal antibodies to a group of new dioxin haptens synthesized in Dr. Hammock's laboratory. These studies also have utilized low-energy molecular models of the polychlorinated dioxins and furans.

IMPACT/TECH TRANSFER A: Hal. This immunoassay methodology is being transferred to FSIS scientist, St Louis. These antibodies have also been supplied to a private kit manufacturer who is evaluating them for their application to a commercial immunoassay following a request from the drug manufacturer for a rapid test. A number of manuscripts describing this assay have been published and the current joint collaborative efforts with FSIS will help establish the usefulness of immunoassays both as rapid detection devices but also in helping federal agencies meet targeted reductions in the levels of organic waste generated by traditional analytical methods. The economic impact of this assay can be estimated simply by comparing the time needed for analysis using the ARS ELISA versus the HPLC method. Analysis of 1000 samples by the HPLC method are calculated to require 6 man-months of effort versus 6 man-weeks for analysis of the same 1000 samples using the ARS ELISA. Likewise the traditional HPLC method would generate in excess of 100,000 mL of organic waste versus no organic waste for analysis of the same samples using the ARS ELISA.

Cef. This immunoassay was developed and evaluated as part of a CRADA. A patent for the monoclonal antibody is pending and the antibodies recently have been licensed to an immunoassay kit manufacturer, and it is currently being formatted into a commercial immunoassay kit. The ability of these antibodies to distinguish and identify cephlasporins from betalactam antibiotics will greatly improve the ability to identify and document the presence of cephalosporin residues in foods. Manuscripts describing the assay have been published.

HyB. A patent was recently issued covering the anti-hygromycin monoclonal antibodies and they have been licensed by ARS to a private kit manufacturing company. Initially this company will target the assay for the analysis of hygromycin in grains, however, it readily can be adapted for analysis of meat samples should a need arise for such a rapid assay. Analysis of grains is necessary since this compound is extensively used in animal production and has a specified withdrawal time that is based in part on the level of drug present in the feed. Thus,

drug levels in grain must be monitored to document that the proper dose is being used. Manuscripts describing these antibodies and their application have been published in the scientific literature.

Flu. Rapid tests such as this one should help producers, as well as government agencies, screen poultry products for the presence of sarafloxacin residues. In addition, manuscripts describing the antibodies have been published in the scientific literature.

44D. Upon successful generation of a suitable immunogen and production of monoclonal antibodies, they will be incorporated into a simple immunoassay by our CRADA partner and be available as a kit for rapid diagnostics of this drug.

SD. Development of the above immunoassays was initiated in collaboration with a CRADA partner. Sulfa drugs are widely used in animal agriculture and method for rapid detection of the most common sulfa drugs would significantly improve the industries' ability to monitor these drugs. Manuscripts describing this work have been prepared.

QC. Development of rapid tests for analysis of dioxins in animal fats would be highly desirable since present technology is time consuming and costly (in excess of $1500/sample). A rapid immunoassay with sufficient sensitivity and a specificity to the most toxic isomers would greatly improve the ability of industry and government regulators to monitor substantial numbers of samples for these highly toxic compounds

OBJECTIVE B: Develop methodologies for production of monoclonal antibodies using recombinant DNA techniques and cost-effective methods for expression of such recombinant antibodies (rAB) in bacterial, or mammalian cell systems.

PROGRESS B: Recombinant antibodies (rAB) are produced by cloning the genes encoding the heavy and light chains of antibody molecules. Briefly, the genes are cloned, modified and then a heavy-chain and a light-chain gene are inserted into an appropriate expression vector. Expression of the resultant rAB molecules is generally done in bacterial or Eukaryotic cell vectors. The advantages of rAB versus traditional antibodies are numerous and include the ability to genetically alter the rAB molecules to alter the specificity and sensitivity of the antibody molecule using simple and rapid recombinant DNA techniques. In addition, rAB methods greatly lessens the reliance on the use of animals for production of new, novel immunochemical reagents. We have chosen a set of anti-dioxin monoclonal antibodies to serve as a model system for development of these methods and to serve as a test case for the ability of rAB to bind small molecules such as pesticides, drugs or toxic chemicals. We have successfully constructed a generic plasmid vector that can accept modified antibody genes and be used to express the antigen binding portion of the antibody molecule, the FAB fragment. Genes from two different anti-dioxin monoclonal antibodies have been cloned using Polymerase Chain Reaction (PCR) methods. Recombinant FAB fragments from both antibodies have been expressed and have been shown to have affinities and specificity for various dioxin congeners comparable with the parent monoclonal antibodies. In addition, using standard DNA methods, the heavy and light chains of these antibodies have been mixed to produce hybrid molecules containing the heavy chain of one antibody and the light chain of another antibody. Significant

improvement in affinity (5-fold) was observed for one of these shuffled recombinant antibodies. We anticipate introducing a number of point mutations into these genes in an effort to improve antibody performance. Previously, we have generated computer-assisted molecular models of the dioxin combining sites for these antibodies and we anticipate generating such models for the hybrid antibodies and mutated rAB. These latter studies will allow us to better understand how to improve specificity and affinity of antibodies that are being used to detect pesticides, drugs and other small toxic chemicals.

IMPACT/TECH TRANSFER B: These molecular studies are only now coming to completion. Their impact will be to modify the way we produce immunochemical reagents in the future. Using these techniques it may be possible to greatly shorten the time needed to produce immunochemical reagents by simply modifying preexisting antibodies or by using these techniques to more efficiently screen larger numbers of clones. Finally, they hold the promise of allowing continuous production of immunochemical reagents using greatly reduced numbers of vertebrate animals.

OBJECTIVE C: Development of molecular imprint resins for extraction, cleanup, and detection of residues in foods.

PROGRESS C: The development of molecular imprints as tools in analytical chemistry is a new and exciting area of study. We have generated a series of molecular imprints including imprints to, atrazine, salinomycin, halofuginone, and ceftiofur. We have studied the application of these imprints as stand-alone assays, comparing them to antibody methods, and as chromatographic supports. Recent studies demonstrated that molecularly imprinted polymers were highly effective reagents for rapid, one-step, selective cleanup and concentration of residues from complex biological matrices (chicken liver samples). This approach, designated molecularly imprinted solid phase extraction (MISPE), improved recoveries of atrazine residues from chicken liver samples as measured using either an HPLC or an ELISA method. We anticipate future studies aimed at incorporating MISPE into an automated, multiple column high pressure liquid handling system capable of utilizing on-line immunoassays for final detection.

IMPACT/TECH TRANSFER C: These studies have been published in scientific journals, chemical industry trade magazines, and presented at major scientific meetings. In addition, we have supplied imprinted polymers to collaborators in Russia who are developing fluorescent-based methods for residue detection.

OBJECTIVE D: Produce food-animal tissues and body fluids containing specified levels of incurred residues of veterinary drugs, pesticides, or other chemicals for use by FSIS in the development and validation of analytical methods.

PROGRESS D: Two studies involving incurred drug residues in animal tissues and fluids were completed. Tissues with incurred residues of sulfmethazine, sulfadimethoxine, and sulfathiazole

in pigs were produced. Liver, kidney and muscle tissues recovered were used for validation of in-house immunoassay methods. In addition, poultry and bovine tissues and fluids with incurred residues of danofloxacin and enrofloxacin were produced and are being used to develop extraction methods compatible with the fluoroquinalone immunoassay discussed above in Objective A.

IMPACT/TECH TRANSFER D: The main impact of these projects was to supply tissues with incurred residues to validate the immunoassays described above. This is a critical component in the development of an immunoassay and such tissue samples are generally not available from other sources. In prior years, specific requests from FSIS for additional tissues with incurred residues were received and reports detailing each incurred project were prepared and forwarded along with the samples to the FSIS or the FSIS designated agency. No such requests were received from the FSIS in the past year, however the laboratory stands ready to produce such samples for FSIS should they be required for calibration of their own internal residue methods.

OBJECTIVE E: Produce highly specific monoclonal antibody-based immunoassays capable of detecting enteric pathogens and normal gastrointestinal bacteria in poultry and swine.

PROGRESS E: A number of monoclonal antibodies to normal swine and poultry intestinal bacteria have been produced. These antibodies have been used to develop rapid immunoassays that have been used to enumerate bacterial levels in the defined competitive exclusion cultures developed by the Unit and to track the fate of these bacteria in normal and treated animals. In addition, a number of monoclonal antibodies have been produced to pathogenic bacteria including *E. coli* O157:H7, *Salmonella typhimurium* (phase II i antigen), and *Campylobacter jejuni*. Future studies are planned to evaluate these antibodies in novel immunoassay formats with a goal of developing detection schemes that can detect microbes in real-time with no or only very limited tissue culturing.

IMPACT/TECH TRANSFER E: The results from these studies have direct impact on our understanding of competitive exclusion as an intervention strategy to control pathogens on the farm. This information will greatly aid in development of next-generation competitive exclusion cultures for pathogen control. In addition, real-time immunoassays for pathogens would have direct applications to the food animal industry.

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METHODS DEVELOPMENT FOR ANALYSIS OF RESIDUES IN MEAT AND OTHER AGRICULTURAL COMMODITIES

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OBJECTIVE A: Develop quantitative multiresidue methods of analysis for chemical residues in meat and other agricultural products suitable for regulatory purposes, emphasizing the use of chromatography, ion-trap mass spectrometry, supercritical fluid extraction, pressurized liquid extraction, and capillary electrophoresis to maximize recoveries of analytes while minimizing or eliminating matrix interferences.

PROGRESS A: Ion-Trap MS Analysis of Meat. A traditional method of extraction of organochlorinated pesticides in meat was modified and streamlined using liquid-liquid partitioning, Florisil clean-up, and gas chromatography/ion trap mass spectrometric detection (GC/ITD). The method is rapid, uses no chlorinated solvents, and requires only a single evaporation step. The detection limits for 10 representative organochlorine and pyrethroid pesticides were below regulatory action levels, and GC/ITD permitted quantitation and confirmation of pesticides with a single injection. Also, the enhanced selectivity of GC/ITD versus traditional selective detectors helped avoid matrix interferences that can trouble conventional detection methods. Additional work has shown that the approach is useful for many other pesticides in the meat matrix, including organophosphates.

Supercritical Fluid Extraction. Investigations of supercritical fluid extraction (SFE) and GC/ITD expanded the number of commodities and pesticides that have been evaluated with the multiresidue approach in nonfatty foods. In all, more than 170 pesticides in over 25 types of fruits, vegetables, grains, and spices have been tested with the SFE approach by the ARS lab, collaborators, and associates. Three traditional methods of extraction using organic solvents (acetone, acetonitrile, and ethyl acetate) were compared with SFE using GC/ITD analysis of 60 diverse pesticides in fruits and vegetables. Side-by-side comparisons of results have consistently shown good agreement between SFE and the traditional methods for most commodity/pesticide combinations. There have been many instances when the SFE method provided higher values for incurred residues in real samples which indicates that the traditional method may not extract bound residues as well as SFE. Each method has certain advantages over the other methods tested, but the significantly lower costs, ease of use, reduced labor, and many other benefits of the SFE and GC/ITD approach make it the more efficient method overall.

Sample Homogenization. A potential drawback with the SFE method was the use of a small sample size. The SFE and GC/ITD approach gives lower detection limit for many pesticides than traditional methods due to the high degree of selectivity in the extraction, but the 2-3 g sample size in SFE may not be representative of the larger sample unless an adequate sample homogenization is employed. A newly introduced commercial chopper was evaluated to provide accurate subsampling of larger, representative food samples for SFE. In the study, 75 diverse pesticides were inhomogeneously fortified in pears. The chopper, which contains a 6 L and a 1 L bowl for different sample sizes using the same device, provided subsamples (1:1 pear;drying agent) with high recoveries and average reproducibility of 8% relative standard deviation for the pesticides.

Analysis of Herbicides in Soybeans. A method of analysis for multiple polar herbicides in soybeans using pressurized liquid extraction and capillary electrophoresis was developed. To obtain extracts sufficiently clean for UV detection of the herbicides at U.S. tolerance levels, a number of time-consuming and labor-intensive clean-up steps were required. In collaboration with EPA scientist, Alex Krynitsky, the procedure was reduced to two simple clean-up steps prior to analysis with liquid chromatography/mass spectrometry (LC/MS) using electrospray ionization. The detection limits were lower with the LC/MS method, and confirmation of the identity of the herbicides was performed at the same time as quantitation. Previously, only inefficient single analyte methods were available for these type of pesticides in agricultural crops, and due to the complexity of the methods and practical constraints of laboratories, these pesticides have not been monitored. With the use of effective multiresidue methods, monitoring for regulatory enforcement risk assessment purposes becomes feasible.

IMPACT/TECH TRANSFER A: The SFE pilot study for multiresidue analysis of pesticides in fruit and vegetables continues in the Pesticide Data Program. The North Carolina Dept. of Agriculture implemented the SFE and GC/ITD method for several commodities in their routine analyses. Beech-Nut Nutrition Corp. (Canajoharie, NY) and Campbell's Soup Company (Camden, NJ) have also implemented the SFE and GC/ITD method for several raw and processed agricultural products. Regulatory laboratories in Australia, Denmark, Israel, Japan, South Korea, Israel, Spain, Sweden, The Netherlands, United Kingdom and other countries have been provided with standard operating procedures for the SFE and GC/ITD method and plan to evaluate the approach. These methods should be of interest to APHIS also. Dr. Lehotay has become Associate Referee in an AOAC International collaborative study, Pesticides in Nonfatty Foods Using SFE and GC/Ion Trap MS.

The capillary electrophoresis and LC/MS methods developed for the polar herbicides in soybeans were transferred to USDA GIPSA FGIS Technical Center, Kansas City, MO, for use in their programs. Soybeans are a very important U.S. agricultural export, particularly to key Asian markets. The new methods developed under this project should facilitate the exporting process by providing a more efficient monitoring procedure for pesticide residues in the commodity.

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DISPOSITION OF BETA-AGONISTS IN FARM ANIMALS.

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OBJECTIVE A: Determine the metabolism, distribution, excretion, and elimination properties of beta-adrenergic agonists in food producing animals.

PROGRESS A: The biotransformation of clenbuterol HCl has been studied in calves. Three pathways of metabolism have been characterized, specifically glucuronidation, oxidation of the aromatic amine, and oxidation and conjugation with glycine to form a hippuric acid analog. Aromatic amine oxidation leads to the formation of arylhydroxyl amine-, arylnitroso amine-, and arylnitro-clenbuterol compounds. These metabolites are unstable and may degrade to parent clenbuterol. Sufficient mass of clenbuterol glucuronide has been isolated to determine its bioavailability in rats.

A residue depletion study in poultry was conducted in which .5, 1.0, and 2.0 ppm dietary [^{14}C]clenbuterol was provided to 36 cockerels for a 2 week period. Four birds from each dietary [^{14}C]clenbuterol level were slaughtered after withdrawal periods of 0, 7, or 14 days. Total radioactive residues (TRR) in edible and non-edible tissues were dose dependent and in edible tissues were greatest in liver. TRR were not completely depleted from liver, adipose tissue, or skeletal muscle by 14 days of withdrawal. Parent clenbuterol represented 21.9 to 48.0% of the TRR in liver and kidney at 0 withdrawal; however, parent clenbuterol was below the limit of quantification (1 ppb) in the liver and kidney after 7 and 14 days of withdrawal. Stereochemical analysis of parent clenbuterol remaining in liver at 0 withdrawal indicated that it was composed primarily of the S-stereoisomer (S:R ratio: 2.8). Insufficient tissue remained for the stereochemical composition in kidney to be determined.

IMPACT/TECH TRANSFER A: These studies have identified metabolites of clenbuterol that could be converted back to parent clenbuterol in the gastrointestinal tracts of recipient animals and which could contribute to toxicity during consumption of contaminated tissues. Studies will be conducted to determine whether these metabolites are available systemically in non-ruminants after oral or gastrointestinal administration in test animals. Studies in chickens have demonstrated that the inactive (+) stereoisomer of clenbuterol comprises the bulk of the parent clenbuterol residue in liver. In cattle, the R (-) stereoisomer was the major metabolite remaining in liver. The predominance of the S (+) isomer would tend to lessen the risk of poisoning when consuming contaminated livers and the predominance of the R (-) isomer would tend to increase the risk of poisoning when consuming contaminated livers.

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DIOXINS IN BEEF, MILK, AND FORAGE

ARS Contact Persons:
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CRIS number: **5442-42000-001**
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OBJECTIVE A: Identify and quantify residues of chlorinated dioxins and furans in beef, chicken, and in animal feeds, in particular, forages. Devise strategies for minimizing the occurrence of these residues in order to minimize the impact on the consumer of meat products.

PROGRESS A: The United States Environmental Protection Agency (EPA) has designated beef as a major contributor to the human dioxin burden because animals grazing on forage contaminated by fallout from burning processes would store these lipophilic materials in adipose. FSIS is also very interested in dioxin levels in beef because of their food safety concerns. The survey was designed to provide data on the dioxin burden that the population receives from consumption of beef. Analyses of domestic beef samples in a study by FSIS/EPA and in our geographic survey generally showed low concentrations of dioxins and furans (often at the non detect level); however, some animals had concentrations that were many times greater than others at a given site. Also, the control animals in a feeding study conducted at Carrington, ND had concentrations of some of the higher chlorinated congeners that were equal to those of the experimental animals, indicating a source of contamination that equaled or exceeded our feeding amounts. Analyses of components in the feeding facilities strongly suggest that the major source of dioxins in animals with high levels is wood (i.e. support posts and dividing planks) treated with pentachlorophenol. Analyses of components from feeding facilities from two sites that produced animals with the greatest concentrations (Oregon State University and Penn State University) also had posts and planks with high concentrations. All of these facilities had been built in the early 1970s. The animals from our feeding study yielded samples having dioxin concentrations sufficiently above non detect levels to determine the distribution of dioxin and furan congeners among several lipid containing matrixes (serum, back fat, perirenal fat, rib eye and liver). On a weight basis back fat and perirenal fat have the highest levels of the lower chlorinated congeners while liver has the highest levels of the higher chlorinated congeners. On a lipid adjusted basis the tetra isomers are evenly distributed (within a factor of two) among the tissues analyzed, whereas the higher chlorinated isomers are found predominantly in the liver. Back fat and perirenal fat appear to be equally suitable tissues for monitoring levels of the lower chlorinated congeners with perirenal fat being the more suitable for lean animals where back fat is often scarce or non existent.

Tissues from the dioxin/furan feeding study had levels that were sufficiently above the limits of detection to allow fairly accurate determinations of the effects of cooking on dioxin/furan concentrations in edible beef tissues. Tissues from both dosed and control animals had high concentrations of the higher chlorinated congeners, but only tissues from the dosed animals had high concentrations of the lower congeners. Cooking of these tissues indicated that dioxins and furans were decreased by approximately 45% , regardless of the concentrations, provided that the fats and juices were discarded. Unaccountable losses were greater for the lower chlorinated dioxin congeners than for the higher chlorinated congeners. The opposite was found for the furan series, suggesting that volatility was the more important factor in dioxin losses while degradation was the dominant factor in furan losses.

Studies with dairy cows and humans have shown that more hepta and octa dioxins are often excreted than consumed. To address issues in these studies and to provide data that may explain why animals in our feeding study and in our geographical survey had very high liver concentrations of the higher chlorinated dioxin congeners, we fed rats pentachlorophenol (PCP) of varying degrees of purity. Liver concentrations of octa and hepta dioxins in control rats and rats fed highly purified PCP were very low. Liver concentrations in rats fed reagent grade PCP were moderately high, and were very high in livers of rats fed technical grade PCP. These data suggest that in rats little or no PCP is converted to dioxins, and that subjects that excrete more octa and hepta dioxins than they consume do so by converting predioxins chlorinated phenoxy phenols) to dioxins.

Our group was involved in the trace back study of chickens contaminated with dioxins by ball clay in feed. Fat from chickens bought at two Fargo, ND supermarkets had less than 0.1 part per trillion of 2,3,7,8-tetrachloro-p-dibenzodioxin while 19 of 33 samples of chicken fat collected from producers in Arkansas, Louisiana and Texas had concentrations ranging from 14 to 20 ppt.

IMPACT/TECH TRANSFER A: Two major sources of dioxin contamination in foods from animal sources have been found to be due to routes other than the commonly accepted smoke stack to forage to animal route. High dioxin/furan levels in beef have been associated with animal exposure to pentachlorophenol treated wood. High levels of dioxins in chickens have been traced to ball clay that has been added to soybean meal as an anti-caking agent. Identification of these sources allows modification of production procedures to suppress the impact of dioxins in humans.

OBJECTIVE B: Study the metabolic disposition and excretion of dioxins in animals so that it can be used to help minimize the occurrence of residues in mammalian tissues.

PROGRESS B: A metabolism study of 1,2,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in a calf was conducted to complement the metabolism studies already conducted in the rat. A single bull calf was dosed orally with the radiolabeled (^{14}C) congener at a rate of 1.2 mg/kg. All the urine and feces was collected at 24h intervals for 4 days. In the calf, as in rats dosed at the same level,

most of the dose was excreted within 4 days (92.0% vs. 94.7%). Feces was the major route of excretion in both systems (81.5% in calf and 77.6% in the rat). Total tissue residues were low in both species and no significant differences were noted in TCDD tissue distribution between the species. In the calf, the large intestine and liver contained the highest levels of the dose, 0.5% and 0.2%, respectively. The highest tissue levels in the rat were in the intestinal tract (0.4%) and the liver (0.2%). The major metabolite identified in the feces was the NIH shifted 2-OH-1,3,7,8-TCDD. Metabolism was necessary for excretion of TCDD in the urine. The major metabolites identified in the calf urine were 2-*O*-glucuronide-1,3,7,8-TCDD, 1-*O*-glucuronide-4,5-dichlorocatechol, and 1-*O*-glucuronide-2-*O*-sulfate-4,5-dichlorocatechol. These urinary metabolites were also present in rat urine following an oral dose of 1,2,7,8-TCDD. The metabolism of TCDD is thought to involve an arene oxide intermediate which is responsible for the NIH shifted product. This arene oxide system must be tightly coupled to a dioxygenase/peroxidase enzyme system to produce the 4,5-dichlorocatechol metabolites; no diphenyl ether intermediates were found. No evidence was obtained from either species that the common detoxication pathway for halogenated aromatic hydrocarbons, i.e. the mercapturic acid pathway, was utilized by either the calf or rats.

The adsorption, disposition, metabolism, and excretion of two non-toxic dioxin congeners (1,3,7,8-TCDD and 1,4,7,8-TCDD) have been studied in ruminating calves and results have been compared to previous studies of these same congeners in laboratory rats. Two calves were orally dosed with either ^{14}C-1,3,7,8-TCDD or ^{14}C-1,4,7,8-TCDD at the 2 mg/kg level. Urine and feces were collected separately in 24 h periods over four days. After four days 107% and 94% of the dose, respectively, had been excreted from the calves. The major route of excretion was feces which cumulatively contained 85-100% of the dose. Urine from both calves contained less than 1% of the dose during the four days of collection. Excluding the GI tract and contents, none of the tissues sampled contained a significant amount of ^{14}C residue. Liver, a primary depot for toxic dioxins, had only 0.02% of the dose remaining after four days while the carcass had < 0.5% of the dose. Preliminary metabolism studies showed 15-40 % of the fecal radioactivity was parent compound. The major metabolites appeared to be the same monohydroxy dioxins which were isolated from the studies in rats. While on-going studies will further investigate the metabolism in calves, these non-toxic dioxin congeners appear to be rapidly eliminated and extensively metabolized via the arene oxidation pathway in ruminating calves similar to what was found in rats.

IMPACT/TECH TRANSFER B: The results show that the less toxic dioxin congeners 1,2,7,8; 1,3,7,8; and 1,4,7,8-TCDD, were rapidly eliminated in both the laboratory rat and the calf. Tissue residue levels, distribution, excretion patterns, and metabolites identified were comparable in the two species, and lead to the conclusion that the rat may serve as a suitable model for the calf in future dioxin metabolism studies with the toxic congeners. The major metabolite identified in the 1,2,7,8-TCDD study, i.e. 2-OH-1,3,7,8-TCDD, has previously been shown to act as a highly competitive ligand of the human thyroid hormone transport protein, tranthyretin (Lans, M. C. *et.al.* 1993. "Structure-dependant, competitive interactions of hydroxy-

polychlorobiphenyls, -dibenzo-*p*-dioxins, and -dibenzofurans with human transthyretin." *Chem.-Biol. Interact.* **88**, 7-21). These data may provide preliminary evidence that dioxin congeners that display no overt signs of short term toxicity may act as endocrine disrupters.

OBJECTIVE C: Develop alternative less expensive methods for the detection of dioxins in food.

PROGRESS C: Polyclonal antibodies from chickens were used to make immunoaffinity columns (IAC), which have been shown to bind 1,3,7,8-TCDD and 2,3,7,8-TCDD. Raw serum or milk spiked with ^{14}C-1,3,7,8-TCDD was applied to the IAC and showed 40% and 4% of the radioactivity, respectively, retained by the column. Extensive dilution of the samples (1:20 for the serum and 1:50 for the milk) increased the binding efficiency of the IAC to 75% and 55%, respectively, but considerably increased the application time. An alternative method of pre-affinity sample preparation was ethanol/hexane partitioning followed by solid phase extraction on a Carbograph cartridge (Alltech Associates, Inc. Deerfield, IL). Removal of interferences (proteins and lipids) in this manner improved the IAC binding efficiency to approximately 90%, but generated several more steps in the overall procedure. Preliminary work with an IAC generated from monoclonal antibodies showed binding efficiencies which may require only minimal pre-affinity preparation. The use of IAC for the clean up of dioxins from serum or milk matrices appears promising as it reduces the amounts of organic solvents required and may decrease the overall time spent on preparing samples.

In a cooperative study with Hybrizyme Corp. (Raleigh, NC) a yeast bioassay was assessed as a dioxin screening test. Ten dioxin congeners and two polychlorinated biphenyls were tested to generate standard concentration/response curves using the bioassay. The assay showed no response differences between the toxic and non-toxic congeners tested. The sensitivity of the assay measured by the 50% response concentration was found to be on the order of 10 ppb and did not approach that of the high resolution GC-MS methods now used in dioxin analysis. Preliminary methods development indicated that extensive cleanup was needed for the analysis of dioxin-spiked milk samples before application of the bioassay.

IMPACT/TECH TRANSFER C: The cost of analysis for dioxins in foods is prohibitively high for regulatory monitoring. Simplification of either the cleanup procedure or the analysis step for these samples can lead to reductions in cost. Our studies demonstrate that an immunoaffinity column made from polyclonal antibodies may be applied to the cleanup of dioxins in biological matrices such as serum and milk. The use of IAC may lead to less expensive and less time consuming dioxin sample cleanups. A yeast bioassay which was assessed as a screening test for dioxins was found to be non-specific and not adequately sensitive for regulatory analysis.

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NEW TECHNOLOGIES TO IMPROVE AND ASSESS MEAT SAFETY
(Pharmacokinetics of Dioxins and Multiresidue Methods for Antibiotics)

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CRIS Completion Date: **February, 2002**

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OBJECTIVE A: To develop mathematical models of the pharmacokinetics of TCDD and related compounds in beef and dairy animals.

PROGRESS A: Additional samples were analyzed from the study of residues produced in milk and tissues of lactating dairy cows fed pentachlorophenol-treated wood. Concentrations of the 2,3,7,8-substituted dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) in milk were measured 0, 12, and 32 days following the end of administration of PCP-treated wood for 58 days. The decline in concentrations following the end of exposure were consistent with a two-compartment depuration model. The half-lives of the second compartment increased with higher chlorination from tetra- through hexa-PCDDs and PCDFs. This result was consistent with the reports on other halogenated organics. In contrast to expectations, however, half-lives of hepta- and octa- PCDDs and PCDFs were much shorter than half-lives of the less chlorinated congeners. These short half-lives were associated with relatively high concentrations of the hepta- and octa- congeners in the liver. Thus, concentration declines probably reflected clearance from the liver rather than body fat, which is traditionally associated with the slow-clearing compartment.

A quantitative mass balance had been conducted for the last 5 days of the 58-day PCP-treated wood dosing period. It was found that the excretion of 1,2,3,4,5,6,7,8-CDD and 1,2,3,4,5,6,7,8,9-CDD exceeded intake by factors of 1.9 and 3.7, respectively. Intake and excretion of other PCDD/Fs did not differ significantly. The initial hypothesis that the apparent synthesis of HpCDD and OCDD occurred in the rumen was not confirmed with *in vitro* fermentation of the PCP-treated wood by rumen microorganisms. Other possible synthesis mechanisms include the liver with excretion into the gastrointestinal tract, microbial synthesis in the lower gastrointestinal tract, and microbial synthesis post-excretion. The relative roles of potential precursors also must be established.

A cooperative study has been initiated with the EPA to determine the mass balance of PCDD/Fs under normal background exposures. The primary purpose is to determine if the hypothesized feed sources will account for all of the residues in tissues and excretory products. Four cows will be measured at three periods separated by approximately 60 days. The first collection period, which included collection of samples of all feed ingredients, drinking water, milk, blood serum, feces, and

urine has been completed. Initiation of the analytical work has been delayed because efforts of the collaborating EPA laboratory have been diverted to another high priority project.

IMPACT/TECH TRANSFER A: These findings, as well as previous work on related research, were utilized in providing information and advice to SIS, EPA, and FDA on characterization and management of dioxin residue problems in poultry, catfish and other species caused by use of contaminated clay as an anticaking agent in soybean meal.

OBJECTIVE B: To develop simple and rapid physical-chemical procedures for detection and confirmation of antibiotic residues in animal products at levels of concern to regulatory agencies, and verify methods using incurred residues from treated animals.

PROGRESS B: A multiresidue procedure was developed for determination of a variety of B-lactam antibiotic residues in milk and tissues. The method is based on HPLC fractionation of milk or tissue extracts. HPLC fractions corresponding to each analyte of interest were collected. For unknown samples, the fractions were tested for antimicrobial activity using the Delvotest P-mini. Positive samples were further confirmed by HPLC. For HPLC analysis of fractions, conditions were changed from those used in the HPLC fractionation procedure. These included changing the column type, changing the pH or adding ion-pairs to the mobile-phase, combinations of these, or derivatization. The method is suitable for determination of the six β-lactams approved for use in the United States including major metabolites of ceftiofur and cephapirin. The method was also demonstrated with seventeen additional β-lactams, some of which are used in other countries and may be used extra label in the United States. Twenty-five samples from FSIS, mostly liver and kidney, which were classified as containing unknown microbial inhibitors (UMIs) were analyzed by this procedure. Five contained a metabolite of ceftiofur, desfuroylceftiofurcysteine, and four contained penicillin G, only one at violative levels. This is the first time that a metabolite of ceftiofur has been identified in tissue samples from commercial sources.

IMPACT/TECH TRANSFER B: The multiresidue β-lactam method has been made available to FSIS field laboratories. Seventeen copies of a poster describing the procedure were distributed to laboratories around the world. The method is used by Silliker Laboratories and the Dairy Quality Control Institute for Milk Testing. It is the only available procedure for determination of such a wide variety of B-lactam antibiotics in unknown samples.

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NEW TECHNOLOGIES TO IMPROVE AND ASSESS MEAT QUALITY
(Hydrodyne Processing and Meat Color/Temperature/Safety Associations)

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CRIS Number: **1265-41420-001**
FSIS Code: **B3MI03**
CRIS Completion Date: **February, 2002**

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OBJECTIVE A: Determine the effect of hydrodynamic pressure (Hydrodyne process) on retail stability and microbial reduction in beef.

PROGRESS A: Boneless beef strip loins and top rounds were vacuum packaged and treated with the Hydrodyne process (350 g of explosive, 18 in from bottom of tank). A portion of each meat cut was removed and re-vacuum packaged for shipping. Representative meat samples for retail display were removed and repackaged for retail display upon arrival at the laboratory. Samples were taken at three different periods after Hydrodyne treatment: day 7, 17 and 21 for the strip loins and day 10, 17 and 21 for the top rounds. Analysis of pH, purge, thiobarbituric acid-reactive substances (TBARS), aerobic plate count and anaerobic plate count were performed for each sampling period. Panel discoloration scores and Hunter colorimeter values were obtained each day of the retail display period for both cuts. All TBARS (a measure of rancidity) were below 0.4. This is well below 1.0, the point at which rancidity is usually detectable. Extended retail display after an extended storage period increased the amount of TBARS. However, no differences among treatments were revealed for either cut. There was a trend for Hydrodyne-treated strip loins to have lower TBARS readings after the extended retail display. Thus, it appears that the Hydrodyne process does not compromise rancidity, which implies flavor stability. Total plate count (aerobic microorganisms) was reduced in both strip loins and top rounds as a result of being treated with the Hydrodyne process. However, microbial numbers were extremely low (<500 cfu/in^2) throughout the entire storage period. Hydrodyne-treated rounds possessed slightly higher numbers of anaerobic microorganisms (day 10) which did not carry through the retail display. Lean color, surface uniformity and surface discoloration scores revealed no differences among treatments in either cut.

IMPACT/TECH TRANSFER A: Results indicate the Hydrodyne process can tenderize meat with no detriment to product display or shelf stability characteristics.

OBJECTIVE B: Identify mechanisms and develop control procedures to prevent inconsistent cooked color/cooked meat temperature relationships.

PROGRESS B: Persistent pink color in beef patties cooked beyond 71 °C continues to present major problems to food service operations including public schools. High muscle pH (>6.0) often is

responsible for this pink color. Ground beef formulations were processed to represent differences in animal maturity, muscle pH, fat content and hot vs. cold processing. Patties were cooked to either 68.3 or 71.1°C. Patties processed from muscle with high pH (obtained 48 hours post-mortem) displayed more pink/red color at both temperature endpoints than low pH formulations. Reductions in a* values between 68.3 and 71.1°C temperatures were observed only for patties made from hot processed muscle. This may be related to the decrease in pH that occurred during cooking for this formulation. Differences in ground beef formulations typical of those of this study may be responsible for the inconsistent cooked color/internal temperature relationships frequently observed with cooked patties. In any case, controlling muscle pH may be critical if consistent cooked patty color-final internal temperature relationships are to be attained. Efforts to limit the influence of pH on pink color were evaluated by increasing fat content (5, 10, 15, 20, 25%) and thawing patties prior to cooking. While neither fat content nor state of patties when cooked after freezing and thawing exerted major influence on color properties, linear effects in association with increased fat content were higher L* values and hue angles and lower a* values. It appears that high pH beef negates much of the effects of fat and thawing patties on color in cooked patties. More than 18 hours of refrigerated thawing may be necessary to completely eliminate pink color in high pH patties cooked to 71°C.

Cooperative studies were initiated with Michigan State University to assess the potential of triose phosphate isomerase (TPI) as an endpoint indicator of cooking in beef patties. TPI activity decreased substantially in cooked patties between 65.5 and 71.1°C. This related closely with substantial changes in cooked color. Lactate dehydrogenase concentration did not undergo considerable decrease until patties were cooked between 71.1 and 76.7°C.

In light of growing evidence that patties cooked to as low as 55°C may possess premature brown color, FSIS in June 1997 changed the recommendations to consumers to be "use a thermometer instead of color to insure safety." In association with that decision, FSIS with ARS (Beltsville, Athens) initiated a nationwide purchase of various types of ground beef to determine the incidence of premature browning. During the pilot phase of this study, it was determined that if the interpretation of brown color in cooked patties means NO pink color, then the incidence of premature browning is very low (0-5% dependent on the product type) in patties cooked to 57.2°C. If brown color is permitted to have a slight amount of pink color present, then the percent premature brown can be 30-40% among patties cooked to 57.2 and 65.5°C.

IMPACT/TECH TRANSFER B: Numerous conference calls and meetings were held with processors, trade associations, action/regulatory agencies, scientists and consumer groups to transfer new information relative to inconsistent cooked color in beef patties. As a result of this transfer, FSIS in June 1997 issued new information to consumers that stated: "use only a meat thermometer to insure safety in cooking ground beef. In addition, FSIS and ARS initiated a large nationwide collection of ground beef to assess the prevalence of premature browning. An open meeting was held in Washington, D.C. in August 1997 to transfer information regarding the pilot study and gather input for the main study. Numerous conference calls and meetings were held between ARS, FSIS, AMS,

FCS and various trade and industry groups to address problems of persistent pink color in beef patties being used by the National School Lunch Program. Information was transferred that led to directions on cooking and temperature monitoring to be used by school food service operations. In addition, a questionnaire was developed for national distribution to determine the prevalence of persistent pink color and sample analysis will be conducted in school year 97-98 to determine the causes of persistent pink color in school lunch operations.

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POST-MORTEM MUSCLE/MEAT CHANGES THAT AFFECT PRODUCT SAFETY & QUALITY

(Determining Temperature to Which Products Have Been Cooked)

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OBJECTIVE A: Evaluate changes in juice color and residual glutamic-oxaloacetic transaminase activity in beef logs cooked to 79.4°C.

PROGRESS A: Five cuts of beef from three age groups of animals, replicated three times by age group, were fabricated into forty-five beef logs, 16 inches long x 4 inches in diameter. Logs were cooked to an end-point temperature (EPT) of 79.4°C±2°C at a point centrally located 8 inches from the bottom since this was shown previously to be the coldest part of the log during cooking. Logs were immediately chilled after cooking and the 2 inch cube of meat containing the thermocouple was removed, sealed in polyethylene and frozen at -20°C for analysis 3 months post-freezing. Samples will be evaluated for meat and juice color after storage to determine if residual red color remains after cooking and freezing, and to determine the amount of residual glutamic-oxaloacetic transaminase (GOT) activity in the juices for use as an indicator of EPTs.

IMPACT/TECH TRANSFER A: Results will show if red color remains in the meat and juices of beef cooked to 79.4°C, and will prove or disprove the validity of the pink-juices test that is currently used by FSIS inspectors. Effect of animal age and cut of beef on the residual red color of the meat and juices will be shown. Use of residual GOT activity as an indicator of EPT and as an alternative test for the pink-juices test will be evaluated.

OBJECTIVE B: Determine if residual GOT activity in commercially processed poultry products can be used to determine end-point temperatures.

PROGRESS B: GOT activity was determined in numerous poultry products that had been commercially processed by moist and dry heat processes. Differences in activity were not significant ($P<0.05$) by processes but were significant among samples. The addition of condiments such as sauces and marinades did not appear to interfere with the use of the test to measure activities.

IMPACT/TECH TRANSFER B: Residual GOT activities were less than 1,000 SFUs of activity/ml of juice indicating that all products had been processed to EPTs greater than 61.1°C. Interaction with

poultry processors has been initiated to determine the EPTs of commercially processed products, and for use as an integral part of a HACCP program.

OBJECTIVE C: Gas chromatographic-mass spectral (GC-MS) analysis of headspace volatiles of microbial isolates from chicken carcasses.

PROGRESS C: Comparative GC-MS analysis of the volatile organic compounds (VOCs) isolated from the head space of cultures of the predominant microbial isolates from commercially processed chicken carcasses was made. Rapid and repeatable isolates of the VOCs were achieved by solid-phase micro extraction techniques. Capillary gas-chromatographic separations were performed, and mass-spectral identifications of the isolates were made to provide objective indices for evaluation of an "Aroma-scan" instrument (electronic nose) that was being tested.

IMPACT/TECH TRANSFER C: Qualitative and quantitative differences in the VOCs by species provides an objective means to evaluate the "Aroma-scan" instrument. Additionally, chromatographic-mass spectral profiles of the VOCs from the predominant microorganisms on processed poultry provides indicators of spoilage during storage.

OBJECTIVE D: Test a hand-held photoionization air analyzer for use in measuring the volatile organic compounds (VOCs) in spoiled meat products.

PROGRESS D: A Perkin-Elmer Photovac 2020 Analyzer has been obtained and preliminarily tested to determine if the volatile organic compounds (VOCs) emanating from stored meat products can be measured as an indicator of spoilage. Commercial packs of hamburger meat that had exceeded recommended shelf-life and were emitting offensive odors were evaluated. These samples were found to give positive results that increased with time in refrigerated storage.

IMPACT/TECH TRANSFER D: These preliminary tests indicate the possibility for use of instruments such as the Photovac for use by Federal Inspectors, processors and retailers to check the quality of stored meat products. Research will be continued to develop techniques to determine the storage quality of meat products.

OBJECTIVE E: Evaluate Use of Multiple Variables to Estimate EPT of Beef Logs.

PROGRESS E: Beef logs were heated to EPT's near 79.4˚C. Exudates from the logs were assayed for residual glutamic-oxaloacetic transaminase (GOT) as well as Hunter L*, a*, b*, hue (h) and chroma (c) values. The data were fitted to linear multiple regression models using 1, 2, 3, 4, 5, or all 6 of these parameters as independent variables and EPT as the dependent variable. The coefficient of determination (R^2) was used to assess goodness of fit. The R^2 increased to 0.73 as the number of independent variables increased to four (b*, h, c and GOT). Adding more variables did not significantly improve the fit. Thus, 27 percent of the variation in EPT remained in the error term of the model.

IMPACT/TECH TRANSFER E: These results indicate that estimates of EPT can be improved by including multiple independent variables in the model. If the R^2 can be increased to the neighborhood of 0.95, this technology has potential for significantly improving methods for estimating EPT.

OBJECTIVE F: Determine Acid Phosphatase (ACP) activity in consumer-style cooked non-frozen and frozen ground beef patties.

PROGRESS F: In a cooperative study with Eastern, Midwestern, and Western FSIS labs and the ARS Meat Quality lab, Beltsville, MD; acid phosphatase activity was determined in 5 ground beef patty classes [i.e., frozen, cooked from frozen (FCF); frozen, thawed, cooked (FTC); fresh, not newly ground, cooked (FNNGC); fresh, newly ground , cooked (FNGC); fresh not newly ground, stored 4 days 40°F, cooked (FNNGS4C). Three target temperatures were tested (135, 150, and 160°F) to determine if ACP activity could be used as an EPT indicator. SAS® statistical results showed no significant main effects among sample types, or test sample location for ACP activity. However, there was a highly significant ($Pr > F = 0.0001$) EPT effect on residual ACP activity. ACP activity was 12084.2, 3444.4, and 827.7 mU/kg sample for 135, 150, and 160°F, respectively. There were no two or three way interactions among main effect factors of product classes, sample location and EPT.

IMPACT/TECH TRANSFER F: This method has potential for FSIS as a laboratory method for certain cooked ground hamburger patty products. Further, this method is being adapted by a cooperating commercial manufacturer for a quality assurance/HACCP methodology for use by commercial processors of precooked hamburger products.

OBJECTIVE G: Determine myoglobin denaturation in consumer-style cooked non-frozen and frozen ground beef patties.

PROGRESS G: Myoglobin denaturation is being studied in cooperation with HemoCue Inc. to determine if it can be used as an EPT indicator for cooked ground beef (hamburger) patties. The method is based on a rapid biochemical method currently in use to measure hemoglobin in the medical field. Biochemical changes have been made to optimize the test for myoglobin. In the same cooperative study with Eastern, Midwestern, and Western FSIS labs and the ARS Meat Quality lab, Beltsville, MD outlined in Objective F, the myoglobin procedure was evaluated. SAS® statistical results showed significant main effects ($Pr > F = 0.0001$, 0.0016, and 0.0001, respectively) among sample classes, test sample location, and EPT for myoglobin content in extracted hamburger juice. There were no two or three way interactions among main effect factors of product classes, sample location and EPT. Myoglobin level decreased ($P<.05$) as EPT increased with values of 3.02, 2.25, and 1.57 for 135, 150, and 160°F, respectively. Sample location showed greater undenatured myoglobin within the center core of the patty compared to a pooled ground sample including the center and surface meat. Myoglobin content ,expressed as g/dl, was significantly ($P< 0.05$) less only in frozen, thawed, then cooked (FTC) patties. This product appears to show a loss of myoglobin content not related to the cooking effect. Further, research is needed on FTC product to determine the

reason for this lower myoglobin level. These results will allow the use of myoglobin level as an EPT verification method within the tested temperature range except for the FTC hamburger patties.

IMPACT/TECH TRANSFER G: Current research findings indicate that this method could be used by the FSIS Food Chemistry lab, state or local health departments to verify compliance of beef pattie products having been heat processed to 155° F or 160° F required by the Pattie Product Regulation. The method could be used in fast food quality assurance programs to verify FSIS/FDA regulations.

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APPLICATION OF SUPERCRITICAL FLUID TECHNIQUES TO FOOD SAFETY AND NUTRIENT ANALYSIS (Using Binary Gas Mixtures)

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CRIS Number: **3620-42000-013**
FSIS Code: **P1CC01**
CRIS Completion Date: **December, 1998**

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OBJECTIVE A: Develop a supercritical-based cleanup method using binary gas mixtures.

PROGRESS A: Based upon previous studies involving the effect of helium in mixtures with supercritical carbon dioxide (SC-CO_2), nitrogen/carbon dioxide mixtures have been tested to devise an analyte specific-extraction/cleanup that reduces the amount of coextracted lipid matter in the resultant extract. Based upon theoretical considerations, mixtures containing nitrogen between 20-40 mole% at several extraction pressures/temperatures were tested with respect to analyte (pesticide) recovery and fat carryover. Using incurred organochlorine pesticides in adipose tissue, and organophosphorus pesticides fortified in chicken fat, we have optimized the extraction of the above two pesticide classes at 8000 psi and 60-70°C using a 30:70/N_2:CO_2 mixture. Recoveries of the organochlorine pesticides relative to those obtained using liquid solvent extraction and alumina column cleanup were excellent and the method requires **no solvent** for both the extraction and cleanup step. Similarly, by adjusting the temperature, a mixture of five organophosphorus pesticides could be recovered at levels between 80-105% with minimal carryover of fat. Using this approach, direct injection of the above supercritical fluid-extracted/cleaned-up extracts showed no adverse effect on gas chromatographic column efficiency for over 50 injections.

IMPACT/TECH TRANSFER A: Extensive interaction with a gas vendor, Scott Speciality Gases, has provided a commercial source which will provide the above binary gas mixtures for use in analytical laboratories.

OBJECTIVE B: Enhanced detection of pesticides via chemical reactions in supercritical fluid media.

PROGRESS B: Method development is being conducted to couple derivatization reactions during supercritical fluid extraction (SFE) for the detection of pesticides and other toxicants. Initial studies are being conducted on neat analytes to optimize reaction and extraction conditions. Several arbamate pesticides have been reacted by placing heptafluorobutyric acid/pyridine/solvent mixtures into an extraction cell followed by extraction/reaction at 5000 psi and 60°C. Results to date indicate that excellent analyte derivatization can be accomplished on the carbamates as judged from detection of the resultant derivative via GC/MS/MS and/or post column OPA derivatization/HPLC-fluorescent

analysis of the unreacted analyte in the extraction cell. Other derivatization agents are being studied by this method, including extractions of fortified analytes in a sample matrix.

IMPACT/TECH TRANSFER B: The described procedure is expected to yield useful methods for analyte specific assays for FSIS that minimize reagent and solvent useage, reduces analysis time, and simplifies assay procedures .

OBJECTIVE C: Develop and test a subcritical water extraction instrument for the determination of toxicants in food matrices.

PROGRESS C: We redesigned a commercial instrument to permit the extraction of toxicants from food using water as a solvent under subcritical conditions. An Applied Separations Spe-ed unit was reconfigured to allow pumping of liquid water into the instrument module, followed by conversion to the subcritical state, thereby permitting, if needed, extractions to be performed with hot, pressurized water. This unit can also be readily reconfigured for extractions with SC-CO_2, thereby providing the analyst with two **environmentally-compatible solvent alternatives for extraction; CO_2 and H_20.** The above conversion was facilitated by decoupling the spool assembly from the pump normally used in the Spe-ed unit, adding equilibration coils and a home-made 316 SS sample cell, and remounting the micrometering pressure letdown valves to avoid any temperature effects on the valves. Initial runs on the modified instrument indicate that the unit is working well mechanically. Currently, numerous extractions are being conducted on model pesticide mixtures in order to optimize extraction conditions and to minimize analyte degradation. Upon completion of these studies, this instrument, or another from Applied Separations, will be reconfigured for operation under supercritical water conditions to permit the destruction of laboratory wastes. Such a bench top unit is expected to be of interest to analytical and chemical-based laboratories faced with the disposal of smaller quantities of toxic solvents and related chemicals.

IMPACT/TECH TRANSFER C: The above instrument modification is anticipated to be of value not only to residue analysts, but result in potential sales for companies who can convert their SFE instrumentation for service under sub-critical water conditions. The instrumental modification designed to permit operation under supercritical water conditions and is the subject of a CRADA with Applied Separations, Inc. of Allentown, PA.

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APPLICATION OF SUPERCRITICAL FLUID TECHNIQUES TO FOOD SAFETY AND NUTRIENT ANALYSIS (Fat Analysis)

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CRIS Number: **3620-42000-013**
FSIS Code: **P1CC02**
CRIS Completion Date: **December, 1998**

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OBJECTIVE A: Compare and demonstrate equivalence of the supercritical fluid extraction (SFE)-based Nutritional Labeling and Educations Act (NLEA) method for fat determination in meat with an organic solvent based NLEA method.

PROGRESS A: Simpler methods are being investigated to show the equivalence or difference between a SFE-gravimetric-based method and those which involve fatty acid methyl ester (FAME) analysis (NLEA). One of the new SFE-based methods includes analyzing extracts from meat samples by both gravimetric and FAME analysis and has shown, in many cases, good agreement. It has been extended with success to other food matrices such as oilseeds, several bakery products, and NIST standard reference material, #1544. These initial results may lead to the method being run as an AOAC Peer-Validated Method instead of an extensive collaborative study.

In a different but somewhat related study, the previously developed SFE/SFR/GC methodic sequence for determining fat content of meats via FAME analysis has been applied to characterize the lipid content of 5 different oilseed types previously characterized as part of a AOCS collaborative study. The data generated using the SFE/SFR/GC method compared well with the results from the AOCS study. The AOCS method has been collaborated and is now AOCS Official Method Am 3-96.

IMPACT/TECH TRANSFER A: The above studies further promote SFE as an alternative, organic solvent-free technique for fat analysis on a variety of food products in several industries. A future collaboration with the Leco Corporation is planned to develop "official" methods based on SFE. Using the above methods, we are currently assisting NIST in evaluating a **proposed meat composite** for fat and cholesterol analysis

OBJECTIVE B: Evaluation of enzyme activity levels in the presence of SC-CO_2

PROGRESS B: In order to screen alternative enzymes as candidates for the previously reported SFE/SFR/GC scheme for generating FAME for NLEA-based fat analysis, we have developed an automated method using the Hewlett Packard Model 7680 extractor for this purpose. Using specified reaction conditions for generating FAME, each extraction cell was loaded with a different enzyme

(lipase) to screen for activity toward performing methanolysis under supercritical conditions. Methanolysis were attempted on three substrates (shortening, phosphatidylcholine, and cholesteryl stearate) in order to provide a general activity screen. Enzymes that were found to have a high degree of activity in performing methanolysis on the above three substrates were Novozyme 435, Chirazyme L-1, and Lipozyme IM. It is interesting to note that enzymatic activity under supercritical fluid conditions did not parallel the corresponding enzyme's propensity to perform hydrolytic cleavage in aqueous buffer solution. These studies provide information on alternative enzymes that can be used in support of SFE/SFR/GC methods for NLEA-FAME-based fat analysis.

IMPACT/TECH TRANSFER B: The above studies provide fundamental data to enable analysts to choose the appropriate enzyme for performing enzyme- assisted NLEA fat analysis. Numerous companies, government agencies, and industry have inquired about the SFE/SFR/GC analysis.

OBJECTIVE C: Develop low solvent supercritical chromatographic methods for the analysis of phospholipids.

PROGRESS C: Several methods to analyze phospholipids using HPLC are reported in the literature. Most of these methods require the use of several solvents and complex programming steps to affect the desired analysis. Recently, using a Hewlett Packard Model 1205 supercritical fluid chromatograph coupled with a Sedex evaporative light scattering detector (ELSD), a new, low solvent SFC method has been developed which separates phospholipid lipid moieties such as: phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidic acid. A Hypersil column (200mm x 4mm; 5 micron) was used at a pressure of 145 bar, 40°C, and a 5% cosolvent elution in SC-CO_2 consisting of 90:10/ethanol:water to affect the rapid separation of the phospholipids. Excellent quantitation relative to established HPLC methods has been achieved using the above method for lecithin mixtures and fractionated, enriched phospholipid samples which are part of a collaborative study. The described assay should be capable of analyzing meat-derived fat samples for phospholipid content using nothing other than a **CO_2, H_2O and ethanol solvent system.**

IMPACT/TECH TRANSER C: The above SFC method is of value to many analysts faced with the difficult task of analyzing phospholipid-containing samples via a simple, low solvent based technique. The above assay also contributes to part of cooperative agreement between NCAUR-ARS/USDA and the Institute of Agricultural Technology at the University of Perugia in Italy.

PUBLICATIONS:

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APPLICATION OF SUPERCRITICAL FLUID TECHNIQUES TO FOOD SAFETY AND NUTRIENT ANALYSIS (Micro Extraction)

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OBJECTIVE A: Apply solid phase micro extraction (SPME) for the analysis of volatiles and semivolatile compounds in foods.

PROGRESS A: Solid phase microextraction (SPME) has been used to measure contaminant marker compounds in fire-exposed meats, aged vegetable oils, and flavor precursors in cut onions. Two fiber types were evaluated for selectivity and the capture of volatile and semivolatile compounds (a polydimethylsiloxane and polyacrylate moiety). Analysis of absorbed compounds on the fiber was performed by GC/ion-trap MS. Results from SPME analysis of the oxidized oil samples paralleled those found in earlier work on similar samples using SFE coupled with GC/MS analysis. Of interest to FSIS was the use of SPME for detecting polycyclic aromatic compounds which are markers for meat exposure to fire and smoke. The SPME technique was able to distinguish between meat samples (smoked chicken, ham, corned beef) that were exposed to fire and smoke and those used as control samples. Using the 100 micron PDMS fiber, the marker compounds identified included: benzene, toluene, xylene, ethylbenzene, butylbenzene, naphthalene and 1-methyl naphthalene.

IMPACT/TECH TRANSFER A: This relatively new technique will better identify volatiles or semivolatile contaminents in meats and similar matrices. The analysis requires **no solvent**, is relatively inexpensive and easy to learn.

PUBLICATIONS:

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NIR SPECTROMETRY TO MEASURE NUTRIENTS AND FOOD PROPERTIES

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OBJECTIVE A: Determine the usefulness of near infrared (NIR) technology for nutrient analysis of meat and poultry products in nutrition labeling and other regulatory samples; and assess these anlyses' effectiveness in reducing the amount of hazardous waste from current laboratory methods.

PROGRESS A: A collaborative study, with 10 laboratories, for proximate analysis of ground beef by near infrared reflectance and transmittance for establishing an approved method protocol, has been submitted to AOAC. This study involves the evaluation of four near-infrared instruments,. The four instruments possess different combinations of the following aspects : reflectance vs. transmittance, short wavelength (850-1050 nm) vs. long wavelength (1100-2500 nm) and scanning vs filter. Data from each instrument model will be treated separately (e.g., four independent collaborative studies.

In the present study the equations for fat, moisture, and protein are furnished by each instrument manufacturer. The collaborative test samples consist of 12 independent samples without proximate data for analysis by collaborators. After equation standardization, the samples will be analyzed by each collaborator. It is assumed that the wide variation in the combinations of aspects will allow future users and manufacturers to estimate the levels of accuracy of their analyses.

All instrument manufacturers have agreed to participate in the collaborative study: 1) Tecator Infratec Model 1265 (transmittance, scanning), NIRSystems Model 6500 (reflectance, scanning), Perten Instruments Model (reflectance, scanning), and Foss Meatspec (transmittance, fixed filter). Golden State Foods in Conyers, Ga. has agreed to supply the ground beef for the study. After the protocol is approved by AOAC, collaborators will be solicited.

IMPACT/TECH TRANSFER A: Results from an AOAC collaborative study will allow current, new users, and instrument manufactures to estimate the level of accuracy of their NIR and NIT proximate analysis. In addition, AOAC approval will allow FSIS to implement this technology for proximate analysis of ground beef.

OBJECTIVE B: Determination of end point temperature (EPT) in cooked ground beef by Near Infrared Reflectance Spectroscopy.

PROGRESS B: NIR is under study (in cooperation with Carl Davis, Poultry Meat Processing and Meat Quality Research Unit, Richard B. Russell Research Center and the FSIS Eastern Laboratory) to determine if it can be used to determine EPT in cooked ground beef patties. Meat juices recovered after cooking patties to an EPT of 135°, 150°, 165°, and 175° F were blotted on 3.7 cm glass micro fiber filters and dried in forced air oven at 50°C for 15 minutes. Near infrared spectra of the dried micro fiber filters were collected from 400 to 2500 nm. Near infrared spectra were related to EPT. The error for determining EPT by NIR was 3.0° F. Near infrared spectra showed a significant difference in protein and color absorbance with increased EPT. Studies are ongoing to decrease sample preparation time and the use of a single or a combination of NIR wavelengths to predict EPT.

IMPACT/TECH TRANSFER B: These preliminary results show that this 15 min. test could have potential as an EPT indicator for verifying FSIS/FDA requirements for fully cooked hamburger patties. In addition, the method with a single or combination of wavelengths could be used in fast food quality assurance programs to verify compliance with FSIS/FDA regulations.

REFERENCE MATERIALS FOR NUTRITIONAL ANALYSIS

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OBJECTIVE A: (1) Development of accurate methodology required to provide reference materials (RM); (2) Choose representative food matrix candidate for reference materials.

PROGRESS A: (1) Development of this project will focus on use of isotope dilution mass spectrometry to provide added information on simple sugars, short chain fatty acids, water soluble vitamins and other organic constituents for Standard Reference Material (SRM); (2) No further research was conducted towards this area of objective A, and will be terminated in the new CRIS project.

IMPACT/TECH TRANSFER A: (1) This research will provide informational content for a series of critically needed food matrix reference materials for use in nutritional labeling regulations analysis by both FSIS and the food industry; (2) This information is being considered for adoption by AOAC INTERNATIONAL to choose matrices representative of all foods for collaborative studies and method validation.

OBJECTIVE B: Define and Establishment of a National Infrastructure to provide RMs.

PROGRESS B: Material to produce a new Canned Meat Standard Reference Material was obtained, with ARS assistance, by NIST in FY97 and characterization will proceed as a FY98 project at NIST. Development of this SRM was proposed by FSIS, through ARS, to address regulatory needs for food labeling of meat products. SRM 1546a Total Diet, a material produced by ARS, became available from NIST in September 1997. Activity continues to establish and develop the Technical Division on Reference Materials (TDRM), AOAC INTERNATIONAL to facilitate availability and use of RMs in method validation, implementation and use of "Official Methods of Analysis". TDRM Liaisons to each of the ten AOAC Methods Committees were established to provide a yearly assessment of needs and activities required for RMs of interest to each Methods Committee. These activities will not be formally recognized in the new CRIS project.

IMPACT/TECH TRANSFER B: These TDRM activities continue progress to establish the necessary national infrastructure required to improve food/nutrition measurements

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NATURAL TOXINS IN POISONOUS PLANTS AND THEIR EFFECTS ON LIVESTOCK

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CRIS Number: **5428-31320-001** **5428-32000-007** **5428-32000-006** **5428-32630-007**

CRIS Completion Date: **September, 1997**

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OBJECTIVE A: Identify toxins in plants and determine clearance times of these toxins from the tissues of animals consuming them.

The mission of the USDA-ARS Poisonous Plant Research Laboratory is to identify plants poisonous to livestock, identify their toxins, describe their modes of action, develop diagnostic procedures, determine the conditions under which poisoning occurs, and develop strategies to minimize or prevent economic losses due to these plants and provide the consumer with quality animal products.

PROGRESS A: The indolizidine alkaloid swainsonine is the toxin in locoweeds (certain species of the genera *Astragalus* and *Oxytropis)*. A study was conducted to quantify swainsonine in tissues of locoweed-poisoned sheep and determine the rate of swainsonine clearance from animal tissues. Swainsonine concentrations in skeletal muscle, heart, brain, and serum were similar at ~250 ng/gm. Clearance from these tissues was also similar with $T_{½}$ of ~20 hrs. Swainsonine at more than 2000 ng/gm was detected in the liver, spleen, kidney and pancreas. Clearance from liver, kidney and pancreas was about $T_{½}$ of ~60 hrs. These findings indicate that poisoned animals have significant tissue swainsonine concentrations and animals exposed to locoweed should be withheld from slaughter for at least 15 days to insure that the locoweed toxin has cleared from all animal tissues.

Certain species of *Lupinus* are important poisonous plants causing toxicity in sheep and cattle. Lupine alkaloids (quinolizidines and piperidines) have been detected in the blood plasma of cattle, sheep and goats after oral consumption of the plant material. Clearance half-life from the serum or plasma was measured to be 12 - 24 hours after dosage of the plant material. Serum elimination times varied for different individual alkaloids. Studies are continuing on the clearance times from other tissues.

Larkspur poisoning (*Delphinium* spp.) is the most important poisonous plant problem in cattle in the mountainous regions of the western United States. The major larkspur alkaloids deltaline and methyllycaconitine (MLA) can be quantified in the serum of cattle and sheep after intravenous injection. The serum clearance half-life was measured for both alkaloids and determined to be between 30 - 60 min after iv dosage. Deltaline and MLA were measured in the urine within 15 minutes after iv dosage.

IMPACT/TECH TRANSFER A: Research on the effects of poisonous plants on livestock has resulted in management strategies that have saved producers millions of dollars. These savings are

vital to the survival of rural ranching communities in many states. This research has improved the diagnosis of poisoned animals, improved the productivity of animals and enhanced the utilization of rangelands where poisonous plants grow.

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